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(54) Title: IMMUNE RESPONSE MODULATOR COMPLEX, AND USES THEREOF			
(57) Abstract			
<p>A method of enhancing antigen presentation is disclosed, wherein an antigen is modified by coupling or incorporation with <math>\alpha_2</math>-macroglobulin (<math>\alpha_2</math>M), a construct thereof, or a reactive fragment thereof. The antigen so prepared is capable of eliciting enhanced immune response from silent, scarce or weak epitopes. This may comprise an actual activation process, a shift in the dominance to a different epitope by reducing recognition of an immunodominant epitope, or another mechanism. Also included are the antibodies which recognize these epitopes, methods of treatment and use, including the preparation of monovalent and polyvalent vaccines, recombinant <math>\alpha_2</math>M constructs, and assay techniques and kits for performing such methods.</p>			

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## IMMUNE RESPONSE MODULATOR COMPLEX, AND USES THEREOF

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5 may have certain rights in the invention.

The present invention is a Continuation-In-Part of co-pending application Serial No. 07/992,899, filed December 18, 1992, incorporated herein by reference in its entirety, to which the above-identified application claims the benefit of priority  
10 pursuant to 35 U.S.C. § 120.

### TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the field of immunology and, more  
15 particularly, to the modulation of the immune response to a variety of antigens, including the enhancement of host immunocompetence and the preparation and administration of vaccines for prevention and treatment of disease states.

### BACKGROUND OF THE INVENTION

20  
Antigen Presentation and Immunogenicity

In general, antigens are "presented" to the immune system by antigen presenting cells (APCs), including, for instance, macrophages, dendritic cells and B-cells in  
25 the context of major histocompatibility complex molecules (MHCs) which are present on the APC surface. Normally, natural antigens and molecules supplied as immunogens are thought to be taken up and partially digested by the APCs, so that smaller pieces of the original antigen are then expressed on the cell surface in the context of MHC molecules.

30  
It is also presently understood that T-lymphocytes, in contrast to B-lymphocytes, are relatively unable to interact with soluble antigen. Typically T-lymphocytes require antigen to be processed and then expressed on the cell surface of APCs in

the context of MHC molecules as noted above. Thus, T-cells, and more particularly, the so called "T-cell receptors," are able to recognize the antigen in the form of a bimolecular ligand composed of the processed antigen and one or more MHC molecules. In addition to presenting antigens on MHC molecules, the 5 APC must be activated to express costimulatory molecules, such as B7/BB1, before effective stimulation of T-cells can occur.

Many epitopes on proteins, including both foreign and endogenous proteins, are generally unrecognized or only weakly recognized by the immune system. These 10 epitopes therefore elicit little or no antibody or other immune response, or at most, only a weak response. It has therefore been difficult and in some instances, impossible to raise antibodies against such epitopes. In contrast, other epitopes elicit extraordinarily strong immune responses, in some instances, to the exclusion (or partial exclusion) of other epitopes within the same antigen molecule. Such 15 epitopes can be termed "immunodominant."

A separate problem arises in the preparation and administration of vaccines, and particularly vaccines that present peptide antigens. Traditional methods for preparing such vaccines that present antigens as macromolecules through 20 conjugation to protein carriers or polymerization are often unable to induce cytotoxic T lymphocytes (CTL) response *in vivo*. In such instances an adjuvant is usually added. Use of an adjuvant in the immunizing protocol has the advantage of enhancing the humoral response but has mixed results in priming specific CTL response. Unfortunately, the most popular adjuvant used in laboratory animals, 25 such as Freund's complete adjuvant, is too toxic and unacceptable for humans. Ideally, protection against viral infection is best provided by both humoral and cell-mediated immunities, including long-term memory and cytotoxic T cells.

For example, the human immunodeficiency virus (HIV), the etiologic agent most 30 closely associated with the acquired immunodeficiency syndrome (AIDS), has become an important objective for various vaccine developments. The

predominant vaccine strategy has focused on the use of the envelope protein antigens gp120 and gp160 of HIV-1 produced by recombinant DNA technology. However, the full promise of their use in vaccines cannot presently be realized unless they are administered along with an effective adjuvant.

#### Adjuvants

An adjuvant should usually be a non-toxic agent that provokes specific responses to antigens. There is a wide spectrum of mechanisms by which an adjuvant 10 functions. It can function by creating a depot at the site of injection that prolongs the release of antigens with antigen-presenting cells. It may also function by activating macrophages to synthesize and/or release costimulatory molecules, cytokines, and other mediators which in turn activate effector T cells or antibody-forming B cells. The net result is that an adjuvant augments specific 15 humoral and cell-mediated immunities with a lower dose of antigen required.

The agents that have been commonly used as adjuvants can be broadly categorized into four groups of which the following two are most significant. The first, and the only clinically acceptable group, comprises the gels of aluminum (e.g. alum) 20 and calcium salts. However, alum is a weak adjuvant and its formulation in laboratory tests of HIV and SIV antigens has been found to be inadequate. The second, and perhaps the most potent group, includes pure compounds and undefined mixtures derived from mycobacterial cell walls. Mixtures such as Freund's complete adjuvant (FCA) and lipopolysaccharides (LPS) are the best 25 known examples. However, FCA and LPS produce side effects. They are pyrogenic and induce arthritis in rats and anterior uveitis in rabbits.

#### Enhanced Antigen Presentation

30 The targeting of Ag to APC has been extensively studied *in vitro* and *in vivo* [For review see (3, 75)]. Techniques that have been used include encapsulating Ag into

liposomes (76,77), crosslinking Ag to antibodies directed against surface proteins (78-82), and forming immune complexes for recognition by FcR (83). A complementary approach of decorating B cell surfaces with mAb recognizing a particular Ag also conferred enhanced ability to present that Ag (84). The

5 capacity for Ag uptake by different ABC appears to correlate with efficiency of presentation (85), although Ag focusing or intracellular signalling may also contribute. In general, targeting of Ag to the APC surfaces appears to enhance the immune response.

- 10 While B-cells possess specific receptors, surface Ig, for capturing the Ag they present efficiently (86,87), macrophages and other non-B APCs must utilize other mechanisms. These may include phagocytosis of particulate or cellular Ag and enhanced endocytosis of opsonized Ag or immune complexes. Yet, the efficient uptake and presentation of soluble Ag by these non-B cell APCs in naive animals  
15 is not fully understood. A receptor-mediated process might be involved.

Among the antigen presenting cells (APC), the macrophages are of particular interest by virtue of the central role that they play in the regulation of the activities of other cells of the immune system. Macrophage act as effector cells in

- 20 microbial and tumor cell killing as well, and are believed to secrete numerous cytokines that orchestrate many of the diverse aspects of the immune response. The ability of macrophage to regulate a range of immunologic events is in part a function of their expression of I<sub>s</sub> surface antigens. The expression of membrane I<sub>s</sub> antigens is essential for the induction of specific T cell responses to antigens  
25 [Unanue (1981), *ADV. IMMUNOL.* 31:1-136].

The effective internalization and processing of diverse proteins forms a central issue in antigen presentation by macrophages. The immune system must balance the capacity for interacting with vast numbers of dissimilar molecules with the  
30 requirements for efficiently responding to very low amounts of Ag. Although macrophages are able to sample their environments through pinocytosis, a need for

more efficient means of internalization, such as a receptor-mediated system, has been suggested (1). The targeting of Ag to surface receptors on macrophages or B-cells, either by artificial crosslinking or by exploiting membrane Ig, enhances the efficiency of presentation (1-3); however, a naturally occurring antigen presentation system in macrophages has not yet been identified.

#### The $\alpha$ -Macroglobulin Family of Proteins

The  $\alpha$ -macroglobulins and the complement components C3, C4, and C5 comprise 10 a superfamily of structurally related proteins. The  $\alpha$ -macroglobulin family includes proteinase-binding globulins of both  $\alpha_1$  and  $\alpha_2$  mobilities. The most extensively studied  $\alpha$ -macroglobulin is human  $\alpha_2$ -macroglobulin ( $\alpha_2$ M), a large 15 tetrameric protein capable of covalently binding other proteins (6-7, 20, 63-68) and targeting them to cells bearing the  $\alpha_2$ M receptor (14, 15, 41, 68). Although size and charge may affect the extent of binding,  $\alpha_2$ M can incorporate proteins and peptides bearing nucleophilic amino acid side chains in the relatively nonselective manner. This rapid covalent linking reaction is restricted, however, to a window of time initiated by proteinase-induced conformational change, during which an internal thioester on each subunit becomes susceptible to nucleophilic substitution 20 (7, 8, 20). Thus,  $\alpha_2$ M, C3 and C4 are evolutionarily related thioester-containing proteins that undergo conformational and functional changes upon limited proteolysis (57, 88), resulting in possible formation of thioester-mediated covalent bonds with targets such as proteinases, cell-surface carbohydrates or immune complexes, respectively.

25

Human  $\alpha_2$ -macroglobulin ( $\alpha_2$ M)<sup>3</sup> is an abundant protein. It consists of four identical subunits arranged to form a double-sided molecular "trap" (4). This trap is sprung when proteolytic cleavage within a highly susceptible stretch of amino acids, the "bait region", initiates an electrophoretically detectable conformational 30 change that entraps the proteinase (5). The resulting receptor-recognized f- $\alpha_2$ M is efficiently internalized by macrophages, dendritic cells, and other cells that express

$\alpha_2M$  receptors [reviewed in (9); see also (69)], which has recently been cloned and sequenced (10, 11). Reaction of  $\alpha_2M$  with methylamine results in a similar conformational change to a receptor-recognized form of  $\alpha_2M$ . Methylamine treated and proteinase treated  $\alpha_2M$  are equivalent with regard to binding, 5 internalization and signalling.

Receptor-recognized  $\alpha$ -macroglobulins from different animal species cross-react with similar affinities for the  $\alpha_2M$  receptor regardless of the proteinase used [See (9, 12, 13) for review]. The additional binding of nonproteolytic proteins does not 10 appear to affect the rate of internalization even when artificial crosslinking is employed (14-16). Therefore, regardless of the mechanism of binding, proteins complexed with f- $\alpha_2M$  can be effectively internalized.

A proteinase-activated antigen capture and delivery system that binds antigens in a 15 nonselective but irreversible manner might be expected to play a particularly important role during the primary antigen exposure *in vivo*. *In vitro* studies, however, do not fully reflect this situation. Unlike T-hybridoma clones, which often do not require costimulatory signals, the responses of naive T-cells encountered during a primary exposure are dependent upon costimulation (70, 71). 20 Furthermore, the relative roles of macrophages, dendritic cells, B cells, and Langerhans cells in mediating *in vivo* immune responses are still unclear. Some studies indicate that soluble antigens may be presented primarily by dendritic cells (72, 73), with macrophages mediating the presentation of particulate antigens or inducing tolerance (74). Thus, *in vivo* studies are necessary to establish whether 25 antigen delivery by  $\alpha_2M$  is sufficient for the induction of fully competent helper T-cells.

The possible role of  $\alpha_2$ -macroglobulin as a delivery vehicle for antigens, hormones or enzymes has been reviewed previously in the art [see Osada et al. (1987), 30 *BIOCHEM. BIOPHYS. RES. COM.* 146(1):26-31; Osada et al. (1988), *BIOCHEM. BIOPHYS. RES. COM.* 150(2):883-889; Ito et al. (1983), *FEBS*

LETTERS, 152(1):131-135; Osada et al., (1987), *BIOCHEM. BIOPHYS. RES. COM.* 142(1):100-106; Osada et al. (1987), *BIOCHEM. BIOPHYS. RES. COM.* 143(3):954-958]. The foregoing articles had urged that the receptor for  $\alpha_2$ M had played a role in the delivery of various proteins. Two references by Osada et al. 5 merit specific mention for their purported teachings.

Osada et al. (1987), *BIOCHEM. BIOPHYS. RES. COM.* 146:26-31 reports that murine T-cell proliferation can be augmented by macrophages fed with an antigen- $\alpha_2$ M conjugate. These workers observed a greater proliferative response to an 10  $\alpha_2$ M- $\alpha$ -galactosidase conjugate than to  $\alpha$ -galactosidase alone. However, rather than using antigen-specific T-cell clones, the T-cells were crudely purified from spleens of immunized mice. Since human  $\alpha_2$ M was used, the enhanced response may reflect xenogenic  $\alpha_2$ M-mediated stimulation. The paper reports no control for 15 this likely artifact. Moreover, there is no evidence that the potent effects of endotoxin, which is a frequent contaminant of cell protein preparations, were considered in this report. Finally, these investigators used a macrophage cell line, rather than primary macrophages, and did not control for possible artifacts that may result from using such a line. The result is a publication of ambiguous data, which requires significant investigation to prove its speculative conclusions.

20 Osada et al. (1988), *BIOCHEM. BIOPHYS. RES. COM.* 150:883-889 reports that antibodies to viral proteins can be produced effectively in response to increased uptake of  $\alpha_2$ M-viral protein conjugate by macrophages in an *in vitro* assay. As with the T-cell proliferation experiment discussed above, this experiment lacks 25 controls. The antibody response to cells stimulated with  $\alpha_2$ M alone, and possible contamination with endotoxins, were not considered. Furthermore, this *in vitro* assay was conducted with unpurified spleen cells and peritoneal exudate cells, leading to a high degree of uncertainty of just what it is that caused the observed effect. The data are also ambiguous with respect to the magnitude of the observed 30 effect. No positive control is shown, and the low maximum OD reported requires further elaboration to be convincing.

Moreover, Ito et al. (1984), *MOL. CELL ENDOCRIN.*, 36:165-173, offers a contradiction in that the findings of these researchers suggested that the effects were independent of any activity relating to the  $\alpha_2$ M receptor.

5

In the past, there have been numerous other studies suggesting a role for  $\alpha_2$ M in immune modulation (Reviewed in (46)). Many studies report an apparently suppressive effect on mitogen-induced proliferation or MLC. Factors complicating interpretation include failure to distinguish between functionally

- 10 distinct forms of  $\alpha_2$ M (47, 48), the presence of uninhibited porcine trypsin that can degrade IL-2 (49, 50), potential contamination with endotoxin (46, 48), which has been shown to suppress Ia expression (51), and the binding of growth regulating substances or lectins to  $\alpha_2$ M, which may also contribute to its apparent effects in assays (21, 46). Different concentrations of  $\alpha_2$ M often yielded paradoxical results
- 15 (21, 46), and some effects attributed to f- $\alpha_2$ M required concentrations several hundred-fold higher than the  $K_d$  for receptor binding. It has been claimed that f- $\alpha_2$ M could oppose the IFN- $\gamma$ -induced Ia upregulation (48); however, it was later demonstrated that this observation was an artifact of the technique used, which emphasized differences in localization of Ia rather than their numbers (52). It has
- 20 also been proposed that s- $\alpha_2$ M functions in host defense against pathogen-derived proteinases, but this has not yet been experimentally confirmed (5). Thus, the role of  $\alpha_2$ M in immune regulation remains undetermined.

A need therefore exists for the development of a more effective and efficient

- 25 antigen presentation strategy that facilitates the development of vaccine formulations that offer improved immunity while avoiding the drawbacks of traditional adjuvant materials.

SUMMARY OF THE INVENTION

- The invention described herein relates to the modulation of the immunogenicity of an antigen. More particularly, the invention relates to enhancing the
- 5 immunogenicity of an antigen. In particular, the present invention relates to modifying the antigenicity or immunogenicity of an antigen by administering a receptor binding form of a  $\alpha_2$ -macroglobulin, in particular  $\alpha_2M$ , or a fragment thereof with the antigen. In a preferred embodiment, a complex between the antigen and  $\alpha_2$ -macroglobulin ( $\alpha_2M$ ) or an active fragment thereof is formed.
- 10 Such complex may be introduced to a cell culture or host, or to a target tissue or organ where it is believed that  $\alpha_2M$  augments presentation of the desired antigen and the development of the corresponding immune response will occur.

The complex of the present invention comprises a covalent binding between the

15 antigen of interest and  $\alpha_2$ -macroglobulin or an active fragment thereof. Further, suitable antigens include nucleophiles, and extend to and include peptides, proteins, carbohydrates, cytokines, growth factors, hormones, enzymes, toxins, nucleic acids such as anti-sense RNA, as well as other drugs or oligonucleotides.

- 20 The presence in  $\alpha_2$ -macroglobulin of a thioester bond which is susceptible to nucleophilic attack when  $\alpha_2$ -macroglobulin is activated by proteinases, ammonia or small amines, is believed to account for the efficient formation of the present complex. Complexes with  $\alpha_2M$  or fragments thereof can be formed with chemical crosslinking agents as well. Moreover, the high affinity that activated  $\alpha_2$ -
- 25 macroglobulin and C-terminal fragments thereof demonstrate for its cellular receptor is believed to account for the efficient presentation of the antigen and significant increase in the speed and magnitude of the immune response that is achieved.
- 30 In a further aspect, the present invention relates to a method for modifying immune recognition of epitopes by the immune system. This may involve

endogenous or foreign molecules (immunogens), and may entail altering the antigenicity thereof or altering the immune response thereto, including without limitation modulating the immune response to (non-modified) naturally occurring endogenous or foreign molecules represented by the immunogen. For example,

- 5     the epitopes of a particular immunogen may be essentially unrecognized, or may be inactive epitopes on otherwise antigenic molecules. Similarly, such epitopes may otherwise be only weakly recognized or responded to by the immune system under normal conditions. Alternatively, such epitopes may be dominantly recognized by the immune system, such that other epitopes on the same
- 10    immunogen molecule do not elicit immune responses. In a particular aspect, the immunogenicity of a T cell epitope containing a strongly nucleophilic residue such as lysine is modified, *i.e.*, decreased, by covalent binding of lysine to the thioester group on the  $\alpha_2$ -macroglobulin.
- 15    One of the advantages of the present invention and a particular feature thereof, resides in the fact that the complex prepared by the covalent binding of  $\alpha_2$ M or its fragments to a given antigen, or the development of the constructs disclosed herein comprising the C-terminal receptor binding regions of  $\alpha_2$ M disposed either singly or in tandem with respective antigens covalently bound thereto, can be
- 20    administered as a vaccine without need for an adjuvant. In fact, and as shown by the data presented later on herein, the immune response achieved by administration of antigen in accordance with the present invention equals or exceeds both *in vitro* and *in vivo*, those levels that would be achieved with conventional formulations including adjuvant. In view of the difficulties that are experienced when adjuvant
- 25    formulations are included in vaccines, the preparation of vaccines in accordance with the present invention represents a significant improvement and offers the promise of a far more efficient vehicle for antigen presentation, and one which will avoid many of the drawbacks such as toxicity and the like that are experienced with current adjuvant-containing formulations.

Also, the complex and/or constructs prepared in accordance with the present invention have particular utility in their direction against macrophage, and other cells that bind or internalize  $\alpha_2M$ . The scope of antigens, immunogens or immune modulating molecules that may be associated in the complex and/or constructs of

- 5 the present invention is equally diverse, as it extends from oligonucleotides, proteins, peptides, cytokines, toxins, enzymes, growth factors, antisense RNA and drugs, to other carbohydrates that may exhibit some desired modulatory effect on the target cells. The invention is therefore contemplated to extend to these variations within its spirit and scope.

10

A further advantage of the invention is that it provides for independently targeting a receptor binding  $\alpha_2M$  or fragment thereof, as well as complexes of the invention comprising these components, for endocytosis or for cell signalling and activation.

Proper activation of the APC is necessary for effective antigen presentation and

- 15 effective stimulation of the immune response in general.

As mentioned earlier, the present invention extends to the preparation of specific constructs, such as fusion proteins, in the instance where a protein or peptide is to be delivered. In such event, fusion protein might be prepared between the C-

- 20 terminal fragment of  $\alpha_2M$  and the protein to be delivered, in which the C-terminal portion of the fusion protein contains the receptor recognition site for the  $\alpha_2M$  receptor. The carboxyl terminal fragment of  $\alpha_2M$  or other  $\alpha$  macroglobulins may be prepared to include a chemically cross-linkable group, such as sulphhydryl groups, so that covalent binding can then be made to the antigen, immunogen, or  
25 the like, of interest for eventual presentation and activation. In such event, the effective concentration of the material being delivered may be potentiated while desirably limiting the total protein concentration. Moreover, the C-terminal construct containing engineered cysteine may be prepared in a tandem arrangement, or with a polyvalent crosslinker attached to it, wherein a plurality of  
30 antigens may be bound by like cross-linking groups. In such instance, one may associate a plurality of diverse, complementary antigens to bind to a corresponding

set of receptors. This strategy may be useful in the preparation and presentation of polyvalent vaccines.

In addition, the constructs of the present invention may be prepared

- 5 recombinantly, by the initial combination of the antigen with a C-terminal fragment, and the subsequent introduction of the resulting construct within a vector for expression in a suitable host. The exact parameters and protocols followed in this preparation are within the skill of a molecular biologist, and will most likely vary depending on the particular antigen, fragment and host.

10

It is contemplated that both positive and negative regulation of the antigenicity of epitopes can be achieved. For example, by rendering epitopes recognized, or recognizable, antibodies can be raised to recognize and bind to the antigen.

Enhanced antigenicity and the ability to raise antibodies to otherwise weak, scarce

- 15 or ineffective epitopes finds great utility not only, for example, in vaccine applications in animals, including humans, but also in producing antibodies which can be used as reagents for, among other uses, binding, identifying, characterizing and precipitating epitopes and antigens, such as the production of antibodies against scarce antigens for research purposes. Preferably, the immunogenicity of
- 20 a given antigen is enhanced according to the methods of the invention.

Alternatively, this invention contemplates the downregulation or suppression of immune responses to immunodominant epitopes, by the preferential stimulation of immune responses to otherwise "subordinate" epitopes, or by the introduction of

- 25 agents or factors that on presentation, would selectively suppress the immunogenicity of the target epitope. This additional ability to modulate antigenicity may be useful, for example, in immunizing animals, including humans, and also in producing antibodies which are reactive towards otherwise silent or weakly antigenic epitopes. Such antibodies are also useful for, among
- 30 other things, binding, identifying, characterizing and precipitating epitopes and antigens *in vivo* and *in vitro*.

Another preferred embodiment of the invention utilizes antigen presenting cells (APCs) and the major histocompatibility complex (MHC) present on the surface of such cells. The antigen is complexed initially with  $\alpha_2$ -macroglobulin or an active fragment thereof, as described above. This complex is then combined with APCs 5 having MHC present on the cell surface as well as receptors for  $\alpha_2$ -macroglobulin, the antigen or the  $\alpha_2$ M-antigen complex, until processing of the antigen is effective for rendering the epitope recognizable. The processed and displayed antigen is then available to react with other components of the immune system which recognize the epitope or the complex-modified epitope in the context of the ABC.

10

The invention described herein also preferably includes the antibodies produced by the methods described herein or in response to the immunogens, modified as described herein, said antibodies including monoclonal, polyclonal and chimeric antibodies, as well as immortal strains of cells which produce such antibodies, for 15 example hybridomas which produce monoclonal antibodies which recognize the molecules and other antigens of interest. Advantageously, such antibodies can be prepared against epitopes on the antigen that are normally secondary or even suppressed.

- 20 The invention also encompasses cellular immune system components, e.g., T-lymphocytes raised in response to such antigens or immunogens, pharmaceutical compositions containing the antigens, antibodies or cellular immune system components and various methods of use.
- 25 The invention provides for enhancing the efficiency of immunizations. This can have useful application not only for potential therapeutic interventions, in particular vaccinations, but also for production of antibodies or primed lymphocytes (T or B) against scarce antigens for research purposes.
- 30 Thus, in specific embodiments, *infra*,  $\alpha_2$ M complexed hen egg white lysozyme (HEL) undergoes enhanced macrophage uptake, processing, and presentation to T-

hybridoma clones *in vitro* compared to free antigen; antibody production in rabbits using two antigens complexed with either human  $\alpha_2$ M ( $H\alpha_2$ M) or a homologous protein purified from rabbit plasma,  $\alpha_1$ -macroglobulin ( $R\alpha_1$ M) was evaluated, and was found that complexing the Ag to  $\alpha_2$ M resulted in 10-500-fold higher IgG titers

5 compared to uncomplexed controls; proteinase-treated  $\alpha_2$ M complexed with insulin protects the antigen from degradation; and a C-terminal fragment of  $\alpha_2$ M that lacks the cis-DPP/oxidation reactive site binds an  $\alpha_2$ M-receptor and induces cell stimulation.

10 Accordingly, it is a principal object of the present invention to provide a method for the modulation of immune response by the administration of a receptor binding  $\alpha$ -macroglobulin or fragment thereof.

15 It is a further object to provide a method for enhancing presentation of particular antigens.

It is a still further object of the present invention to provide a method as aforesaid where particular antigens are directed to the macrophage by means of a complex of said antigens with  $\alpha_2$  macroglobulin or an active fragment thereof.

20

It is a still further object of the present invention to provide a method and corresponding complexes as aforesaid that facilitate improved immune recognition and activation.

25 It is a still further object of the present invention to provide a method and corresponding complexes as aforesaid that can be used to selectively activate epitopes in distinction to other immunodominant epitopes.

30 It is a still further object of the present invention to provide a method for the facile development of clinically significant amount of antibodies directed against scarce antigens.

It is still a further object of the present invention to identify peptides that bind to and activate one or more receptors that bind  $\alpha_2M$ .

Other objects and advantages will become apparent to those skilled in the art from  
5 a review of the ensuing detailed description which proceeds with reference to the following illustrative drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 10 FIGURE 1 depicts the binding of  $^{125}I$ -HEL to different conformational forms of  $\alpha_2M$ . A two-fold molar excess of  $^{125}I$ -HEL was incubated for 30 min at room temperature with 1.4  $\mu M$  amounts of native  $\alpha_2M$ ,  $\alpha_2M$  undergoing PPE induced conformational change, or pre-formed f- $\alpha_2M$ . The PPE was inhibited by addition of 100 mM dichloroisocoumarin after 10 min. Samples were analyzed by  
15 SDS-PAGE under reducing (R) and nonreducing (NR) conditions (A & B) and by native pore limit gel electrophoresis (C & D). Coomassie blue stained gels (A & C) and their corresponding autoradiograms (B & D) are shown. Four reactions were analyzed as indicated: (a) s- $\alpha_2M$  +  $^{125}I$ -HEL, (b) s- $\alpha_2M$  +  $^{125}I$ -HEL + subsaturating PPE (~ equimolar with  $\alpha_2M$ ), (c)  $\alpha_2M$  pretreated for 10 min with  
20 PPE, which was inhibited with dichloroisocoumarin, before addition of  $^{125}I$ -HEL, (d)  $^{125}I$ -HEL + methylamine-treated  $\alpha_2M$ . The arrow represents the position of migration for HEL that is covalently complexed to the C-terminal half of the "bait-region"-cleaved  $\alpha_2M$ , when analyzed after reduction. Molecular weight standards are indicated on the left: denatured but nonreduced  $\alpha_2M$  (360 kDa),  
25 reduced  $\alpha_2M$  (180 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Positions of migration for  $\alpha_2M$  conformational forms were determined using native  $\alpha_2M$  (s- $\alpha_2M$ ) and methylamine-treated  $\alpha_2M$  (f- $\alpha_2M$ ).  
30 FIGURE 2 comprises two graphs:

(A) Sensitivity of  $^{125}\text{I}$ -HEL- $\alpha_2\text{M}$  complex formation to  $\beta$ -aminopropionitrile.

$^{125}\text{I}$ -HEL,  $\alpha_2\text{M}$ , and PPE (molar ratios of 0.3:1:2) were coincubated for 10 min at room temperature in HEPES buffer containing 0-200 mM  $\beta$ -aminopropionitrile. Reactions were terminated and samples analyzed by native pore limit gel

5 electrophoresis (●) or nonreducing SDS gel electrophoresis (■) as described in METHODS. Values are expressed as percent of control incubations in the absence of  $\beta$ -aminopropionitrile, and reflect mean  $\pm$  SD from duplicate determinations. (B) Stoichiometry of  $^{125}\text{I}$ -HEL binding to proteinase-activated  $\alpha_2\text{M}$ . Incubations of  $\alpha_2\text{M}$  (1.4  $\mu\text{M}$ ) and increasing molar excesses of  $^{125}\text{I}$ -HEL  
10 were treated with saturating amounts of proteinase (2-fold for PPE and 1.5-fold for human neutrophil elastase) for 10 min at room temperature. After the reaction was terminated by addition of dichloroisocoumarin, bound  $^{125}\text{I}$ -HEL was separated from free  $^{125}\text{I}$ -HEL on reducing and nonreducing SDS gels or on native gels and analyzed as described in METHODS. The results derived from using PPE are  
15 shown. Total binding (●), total covalent binding (■), and reductant resistant covalent binding (▲) are expressed as the mean  $\pm$  SD from four independent experiments.

FIGURE 3 is a graph of specific uptake of  $^{125}\text{I}$ -HEL derivatives by macrophages.

20 Log dilutions of either  $^{125}\text{I}$ -HEL (●), or  $^{125}\text{I}$ -HEL- $\alpha_2\text{M}$ -PPE complexes (■) were incubated with macrophage monolayers at 37°C for increasing periods of time as described in METHODS. Total cell associated radioactivity was determined, and specific association was calculated by subtracting the nonspecific binding determined from incubations with 100-fold molar excesses of unlabelled HEL or of  
25  $\alpha_2\text{M}$ -methylamine. A representative time course for uptake of 100 nM concentrations of the HEL derivatives is shown. Error bars represent one SD from quadruplet samples.

FIGURE 4 is a graphic presentation of data generated by:

30 (A) Competition for the uptake of  $^{125}\text{I}$ -HEL derivatives.  $^{125}\text{I}$ -HEL- $\alpha_2\text{M}$ -PPE complexes (10 nM) or free  $^{125}\text{I}$ -HEL (10 nM) were incubated with macrophage

- monolayers ( $10^3$ ) for 2 h at 37°C in the presence of 100-fold molar excesses of competitors. Uptake was terminated as described in METHODS. Values were expressed as percent of control incubations lacking competitors (100% = 134 cpm for  $^{125}\text{I}$ -HEL- $\alpha_2\text{M}$ -PPE; 100% = 25 cpm for  $^{125}\text{I}$ -HEL). Error bars represent 5 one SD from quadruplet samples. These results are representative of 3 independent assays done at 10 nM and 25 nM, with pulse lengths of 1 or 2 h.
- (B) Competition for processing and presentation of HEL- $\alpha_2\text{M}$ -PPE complexes by macrophages. Macrophages were pulsed for 2 h with HEL- $\alpha_2\text{M}$ -PPE complexes (20 nM) in the presence of 100-fold molar excesses of  $\alpha_2\text{M}$ -methylamine or BSA.
- 10 After washing, the macrophages were analyzed for the ability to stimulate 3A9 hybridomas as described in METHODS. Presentation was expressed as percent of control incubations lacking competitors (100% = 3572 cpm). Values shown are the mean  $\pm$  SD of results from triplicate samples, and are representative of two independent assays (20 nM and 45 nM).
- 15 FIGURE 5 is a graph depicting the results of antigen presentation by macrophages pulsed with different forms of HEL. Macrophages were pulsed with free HEL (●), HEL- $\alpha_2\text{M}$ -PPE complexes (■), or free HEL in the presence of equimolar amounts of  $\alpha_2\text{M}$ -methylamine (▲), for two h before extensive washing to remove 20 excess Ag. Pulsed macrophages were assayed for their ability to stimulate IL-2 secretion by HEL-specific T-hybridomas as described in METHODS. Values are expressed as mean  $\pm$  SD from triplicate samples, and are representative of four independent assays.
- 25 FIGURE 6 is a graph depicting the time and concentration dependence of presentation to HEL-specific T-cells. Macrophages were pulsed with log dilutions of either free HEL (●) or of HEL- $\alpha_2\text{M}$ -PPE complexes (■) for different time periods ranging from 15 min to 3 h. Pulsed macrophages were assessed for their ability to stimulate IL-2 secretion by 3A9 T-hybridomas. The 24 h time points 30 were obtained from coincubating macrophages and T-hybridomas with the Ag. Supernatants from these all experiments were analyzed for IL-2 activity as

described in METHODS. A detectable response was defined as > 1 SD above the baseline, which was calculated from six incubations with no added Ag. The minimum concentrations of the two Ag forms that were required to achieve a measurable T-hybridoma response are plotted against time. Error bars represent 5 one SD derived from triplicate samples.

FIGURE 7 is a graph illustrating the stimulation of T-hybridomas by macrophages in the continued presence of Ag. Peritoneal macrophages and 3A9 T-hybridoma cells were co-incubated for 24 h with varying concentrations of HEL (●), HEL-10  $\alpha_2$ M-PPE complexes (■), or equivalent concentrations of control  $\alpha_2$ M-PPE complexes (▲). Control curves from similar incubations that lacked 3A9 cells (◆) or macrophages (□) are also shown. IL-2 secretion during this 24 h period was quantified as described in METHODS. Error bars represent one SD derived from triplicate samples.

15

FIGURE 8 denotes a primary IgG response to injections of HEL. Pathogen-free NZW rabbits were injected s.c. with the equivalent of 124  $\mu$ g HEL. Shown here are the rates of substrate hydrolysis (alkaline-phosphatase coupled 2° Ab) plotted against reciprocal dilutions of sera obtained two weeks after the injection. Four 20 rabbits were injected with H $\alpha_2$ M-HEL-PPE complexes in HEPES buffer (■), three with HEL emulsified in CFA (◆), three with free HEL in HEPES (○), two with HEL mixed with s- $\alpha_2$ M (▼), and two with HEL mixed with preformed f- $\alpha_2$ M (methylamine-treated) (▲). The symbol represents the mean  $\pm$  SD. A representative curve for preimmune sera is shown (●).

25

FIGURE 9 depicts anti-HEL IgG titers elicited by rabbit (R)  $\alpha_2$ M complexes compared with H $\alpha_2$ M complexes. Sera were collected from each rabbit weekly after a single injection at Week 0 with the indicated equivalent doses of HEL. The maximum dilution factor which yielded substrate hydrolysis rates of at least 1 30 mOD/min were defined as the end titer. A zero titer indicates that substrate hydrolysis was not detectable in a 100-fold dilution. (A) Mean titers are shown

for three rabbits receiving  $\text{H}\alpha_2\text{M}$ -HEL-PPE complexes equivalent to 50  $\mu\text{g}$  HEL (■); four receiving 50  $\mu\text{g}$  free HEL (○); and two receiving 50  $\mu\text{g}$  HEL mixed with f- $\alpha_2\text{M}$  ( $\text{H}\alpha_2\text{M}$ -methylamine) (▲). (B) Mean titers for two rabbits receiving  $\text{R}\alpha_2\text{M}$ -HEL-PPE complexes equivalent to 40  $\mu\text{g}$  HEL (□); three receiving 40  $\mu\text{g}$  HEL emulsified in CFA (◆); and four receiving 50  $\mu\text{g}$  free HEL (○).

FIGURE 10 depicts anti-PPE antibody responses. Each rabbit was injected s.c. with equivalent to 200  $\mu\text{g}$  PPE. Substrate hydrolysis rates in Week 3 sera are shown in comparison with preimmune sera (□). The rabbits received either  
10  $\text{H}\alpha_2\text{M}$ -HEL-PPE complexes (■), inhibited PPE alone (○), or inhibited PPE + 6 mg of BSA (▼). The symbol represents the mean from two rabbits, and the bars show the high-low range of the values.

FIGURE 11 presents a schematic illustrating a potential role for  $\alpha_2\text{M}$  (and other  $\alpha$ -macroglobulins) in Ag processing by macrophages. The  $\alpha_2\text{M}$  conformational forms are derived from a previously published model, which was based on electron micrographs (4). (1) Diffusion of proteins in and out of the s- $\alpha_2\text{M}$  "trap." (2) Proteolytic cleavage of the  $\alpha_2\text{M}$  "bait" region [See (5)], results in capture of the proteinase (stippled "pacman") and potential Ag (striped polygon).  
20 (3) Binding of receptor-recognized f- $\alpha_2\text{M}$  to specific receptors on macrophage ( $M\emptyset$ ) surfaces (crosshatched orbs), results in rapid internalization, and possibly intracellular signalling. Some unbound Ag may also enter through pinocytosis. The receptor is recycled to cell surface after releasing its ligand. (4) Partial degradation of the antigenic proteins in endosomal/lysosomal compartments (bold circle). (5) Intersection with class II MHC molecules (dotted rectangles) and re-expression of the MHC-associated Ag fragment at the cell surface. (6) Specific stimulation of helper T-cells (TH) in conjunction with costimulatory signals (*i.e.*, CD28/B7), leading eventually to antibody synthesis (solid Y's) by plasma B cells.  
25  
30 FIGURE 12 presents an autoradiogram of a non-reduced SDS-PAGE with labelled insulin. Lanes A-G are described in Example 3, *infra*.

FIGURE 13 presents graphs reporting changes in the level of intracellular calcium,  $[Ca^{2+}]_i$ , observed in TG-elicited macrophages. (A) A representative response of a single cell on addition of methylamine-treated  $\alpha_2M$  (40 nM); in this study, 45 individual cells were evaluated. (B) A representative response of a single cell on addition of the 20 kDa RBF of rat  $\alpha_2M$  (●) (40 nM), or buffer (○); in this study, 250 cells and 15 cells were evaluated, respectively, after addition of RBF or buffer. Changes in  $[Ca^{2+}]_i$  were monitored using the fluorescent  $Ca^{2+}$  indicator dye Fura-2/AM as described (Misra et al., (1993), BIOCHEM. J. 290:885-891). Arrows indicate the time at which  $\alpha_2M$ -methylamine (40 nM) and RBF (40 nM) were added.

FIGURE 14 presents a graph showing the percent change in the concentration of intracellular calcium upon exposure to varying concentrations of either  $\alpha_2M$ -methylamine (■) or RBF (○). Calcium mobilization was evaluated as described in the legend to FIGURE 13.

FIGURE 15 presents graphs showing increased histone-III phosphorylation resulting from exposure of macrophages to  $\alpha_2M$ -methylamine,  $\alpha_1$ -inhibitor<sub>3</sub> and RBF. Histone phosphorylation is a consequence of PKC activity. (A)  $^{33}P$  incorporation after treatment with various combinations of buffer, the 20 kDa RBF from rat  $\alpha_1M$ , and the PKC inhibitor staurosporin. The presence or absence of one or more of these reagents is shown in the Figure. (B)  $^{33}P$  incorporation upon treatment with  $\alpha_2M$ -methylamine (A,B) at 200 nM;  $\alpha_1$ -inhibitor<sub>3</sub> (C,D) at 200 nM; or RBF (E,F) at 40 nM in the absence (A, C E) or presence (B, D, F) of staurosporin.

FIGURE 16 presents graphs showing movement of tritiated [ $^3H$ ]-phorbol dibutyrate ( $[^3H]$ -PDBu) to cell membranes in response to treatment with  $\alpha_2M$ , rat  $\alpha_1$ -inhibitor<sub>3</sub>, and rat  $\alpha_1M$  RBF. Movement of the diacylglycerol analog [ $^3H$ ]-PDBu demonstrates activation of PKC. TG-elicited macrophages were cultured as described. The macrophages were exposed to the ligands for 20 min, after which

they were assayed for PKC activation by binding of  $^3\text{H}$ -phorbol dibutyrate as previously described (Misra, U.K. and Sahyoni, N.E. (1987), *BBRC*, 145:760-767). Duplicate experiments were performed employing  $3.5\text{-}4 \times 10^6$  cells in each study. The values are mean  $\pm$  SEM. (A) PKC activation in response to stimulation with (a) buffer; (b) 200 nM slow- $\alpha_2\text{M}$ ; and (c) 200 nM fast- $\alpha_2\text{M}$  ( $\alpha_2\text{M}$ -methylamine). (B) PKC activation in response to stimulation with (a) buffer; (b) 200 nM slow- $\alpha_1\text{-inhibitor}_3$ ; and (c) 200 nM fast- $\alpha_1\text{-inhibitor}_3$  ( $\alpha_1\text{-inhibitor}_3$ -methylamine). (C) PKC activation in response to stimulation with (a) buffer and (b) 40 nM RBF.

10

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, conventional molecular biology, microbiology, cloning technology and recombinant DNA techniques may be utilized which are within the level of skill in the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.B. Hames & S.J. Higgins ed 1985); "Transcription And Translation" (B.D. Hames & S.J. Higgins eds. 1984); "Animal Cell Culture" (R.I. Freshney ed 1986); "Immobilized cells And Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), the teachings of which are incorporated herein by reference.

25 The following terms and abbreviations are used herein, and have the following meanings unless otherwise specified:

The term "immunogen" refers to any substance, such as a molecule, cell, virus or fragment of such molecule, cell or virus which can be administered to an individual in an effort to elicit an immune response. The term "immunogen" thus simply refers to such substances which are or can be administered or otherwise

used to raise antibodies or cellular immune system components, such as by "priming".

When used in connection with "immunogen", the term "molecule" refers to a  
5 molecule or molecular fragment of the antigen unless otherwise specified.

- Likewise when used to refer to a cell, virus or fragment thereof, the immunogen can be the cell, virus or component thereof, which can be disposed in a complex or construct in accordance with the present invention to enhance the immune  
10 response thereto. The term "immunogen" therefore encompasses antigenic compounds, such as foreign proteins as well as species which are essentially non-antigenic in the absence of the treatment described herein, cells, viruses, and cellular and viral components.
- 15 The term "antigen," which may be abbreviated "Ag," refers to substances, e.g., molecules which induce an immune response. It thus can refer to any molecule contacted by the immune system, and may include without limitation, proteins, nucleic acids and the like, and may even extend to carbohydrates capable of presentation in accordance herewith. Generally, each antigen typically comprises  
20 one or more epitopes.

- Preferably the antigens described herein or epitopes thereon, do not substantially induce an immune response or other immunological reaction upon injection or other exposure to a normal, substantially immunocompetent host. They may also  
25 include scarce antigens that are difficult to obtain or purify, or antigens that require adjuvant or administration in large amounts ( $\mu\text{M}$ ) for efficient immune responses. Based on the foregoing, "antigenicity" and "immunogenicity" are used interchangeably.
- 30 The term "protein" refers to synthetically produced and naturally occurring polypeptides, fragments of polypeptides and derivatives thereof which may

provoke an immune response, either *in vitro* or *in vivo*. For convenience, but not by way of limitation, the description below utilizes the term "protein" but these teachings also apply to other compounds which either contain protein residues or that are otherwise structurally similar. Oligonucleotides, carbohydrates, and 5 amine-containing lipids, as well as other reactive biomolecules may be mentioned as non-limiting examples. The teachings contained herein are therefore not to be limited to proteins or fragments thereof.

The terms "immunocompetent", "normal immune system" and like terms refer to 10 the immune response which can be elicited in a normal mammalian host with the antigen of interest, when the antigen in question is administered without the modifications and preparation described herein. The immunogen can simply be administered to the host in unmodified form, and the normal immune response evaluated. Thus, using art recognized methods, this control is readily ascertained 15 without resort to undue experimentation.

The term "antibody" refers to immunoglobulins, including whole antibodies as well as fragments thereof, such as Fab, F(ab')<sub>2</sub> or dAb, that recognize or bind to specific epitopes. The term thus encompasses, *inter alia*, polyclonal, monoclonal 20 and chimeric antibodies, the last mentioned being described in detail in U.S. Pat. Nos. 4,816,397 and 4,816,567, which are incorporated herein by reference. An antibody "preparation" thus contains such antibodies or fragments thereof, which are reactive with an antigen when at least a portion of the individual immunoglobulin molecules in the preparation recognize (i.e., bind to) the antigen. 25 An antibody preparation is therefore termed "non-reactive" with the antigen when the binding of the individual immunoglobulin molecules to the antigen is not detectable by commonly used methods.

An antibody is said to "recognize" an epitope if it binds to the epitope. Hence, 30 "recognition" involves the antibody binding reaction with an epitope, which may

include the typical binding mechanisms and methods. "Binding" is thus used in the conventional sense, and does not require the formation of chemical bonds.

The term "epitope" is used to identify one or more portions of an antigen or an

- 5 immunogen which is recognized or recognizable by antibodies or other immune system components. The "epitope region", as used herein, refers to the epitope and the surrounding area in the vicinity of the epitope; taking into account three dimensional space. Hence, this may take into account the tertiary and quaternary structure of the antigen.

10

"Processing" and "presentation" refer to the mechanisms by which the antigen is taken up, altered and made available to the immune system. Presentation also includes, when appropriate, complexation or binding with MHC and other molecular events associated with generating an effective T-cell response. In

- 15 certain instances, processing entails the uptake and partial proteolytic degradation of the antigen by APCs, as well as display on the APC surface in the context of MHC.

- 20 The terms "reaction" and "complex" as well as derivatives thereof, when used in this general sense, and are not to be construed as requiring any particular reaction mechanism or sequence.

The abbreviation "MHC" refers to major histocompatibility complex, a series of compounds which is normally present to a greater or lesser degree on the surface

- 25 of, among others, antigen presenting cells. MHC functions to "signal" cellular immune system components, e.g., T-lymphocytes, to recognize and react with the antigen presenting cell and/or the antigen bound to said cell and/or the MHCs thereof. The term "signal" is used in the general sense to refer to the initiation of the reaction between T-cells and APCs bearing processed antigen in the context of  
30 MHC. As such the "signal" may involve any reaction between these components

which causes the antigen to become recognized by antibodies, an antibody preparation or by the cellular immune system components.

- For purposes of the present invention, the term " $\alpha_2$ -macroglobulin" and its abbreviation " $\alpha_2M$ " are to be used interchangeably. Moreover, the use of  $\alpha_2$ -macroglobulin in accordance with the present invention is believed to be more generally applicable to  $\alpha$ -macroglobulins and to the macroglobulin family, and the scope of the invention is to be interpreted in this broader fashion.
- 10 Preferably, the term  $\alpha_2M$  refers to human  $\alpha_2M$ , or a receptor-binding fragment thereof. However, this term includes, but is by no means limited to, rat  $\alpha_2M$  (a homotetramer); rat  $\alpha_1M$  (a homotetramer); rabbit  $\alpha_1M$  (a homotetramer); human pregnancy zone protein (a homodimer); cow  $\alpha_2M$  (a homotetramer); dog  $\alpha_2M$  (a homotetramer); duck ovostatin, or ovomacroglobulin (a homotetramer); hen  
15 ovostatin, or ovomacroglobulin (a homotetramer); rat  $\alpha_1$ -inhibitor-3 (a monomer); frog  $\alpha_2M$  (a homotetramer); as well as receptor-binding fragments thereof.

The term "receptor-binding" refers to the ability to bind to a specific receptor on an APC. The receptor may mediate endocytosis, signalling and cell activation, or  
20 both. It is presently believed that there are two receptors for  $\alpha_2M$ . One receptor mediates signalling, and thus cellular activation and growth. The other receptor mediates endocytosis. A C-terminal fragment of  $\alpha_2M$  induces macrophage activation. When this fragment lacks a cis-dichlorodiamine platinum (cis-DDP)/oxidation sensitive reaction site, it appears to bind to the signalling receptor  
25 but not as well as the endocytic receptor. When the C-terminal fragment includes the cis-DDP/oxidation sensitive reaction site, it appears to bind to both receptors.

Other abbreviations: slow (s)- $\alpha_2M$ , the native conformation of  $\alpha_2M$  that is not receptor-recognized; fast (f)- $\alpha_2M$ , the receptor-recognized forms of  $\alpha_2M$  derived  
30 from treatment with proteinase or with methylamine; H $\alpha_2M$ , human  $\alpha_2M$ ; R $\alpha_1M$ , rabbit  $\alpha_1$ -macroglobulin, the  $\alpha_2M$ -equivalent purified from rabbit plasma; HEL,

hen egg lysozyme; PPE, porcine pancreatic elastase; APC, antigen presenting cell.

In accordance with the present invention, a method for enhancing the presentation, recognition and uptake of antigens is disclosed, which comprises administering

- 5 said antigen with a material selected from the group consisting of  $\alpha_2$ -macroglobulin and an active fragment thereof and plural such active fragments thereof, and directing said  $\alpha_2$ M to the target cellular mass to which presentation of said antigen is intended. Preferably, the antigen is in a complex with the  $\alpha_2$ M or fragment thereof. More preferably, the antigen is in covalently associated with the
- 10  $\alpha_2$ M or fragment thereof. In contrast to naturally occurring complexes of proteins with  $\alpha_2$ M, the present invention advantageously provides for forming complexes comprising substantially a single antigen or a few antigens, rather than a diverse population of proteins. As used herein, the term substantially indicates that greater than 30% of the antigen in the complex is a specific antigen; preferably
- 15 greater than 50%; more preferably greater than 75%; and most preferably greater than about 90%.

More specifically, the active fragments of  $\alpha_2$ -macroglobulin may comprise the carboxyl terminal region thereof and said region including the receptor recognition

- 20 site, and said region having associated therewith chemical cross-linking moieties. Exemplary such moieties would comprise a cysteine residue or other moiety providing a disulfide bond for covalent attachment to the antigens in object.

As discussed in the Background of the Invention section, *supra*, native  $\alpha_2$ M can be

- 25 used to entrap and covalently or non-covalently complex with an antigen of interest. In one embodiment, the trapping and complex formation occur by activation, *e.g.*, with proteolysis.

In another embodiment, fast  $\alpha_2$ M, which is  $\alpha_2$ M treated with an activating agent

- 30 such as ammonia or methylamine, or with proteinase, can be used. This activated form of  $\alpha_2$ M can bind to the endocytic receptor and the signalling receptor.

One  $\alpha_2$ M-R has been purified from human placenta, rat hepatocytes, and human fibroblasts (Ashcom et al. (1990), *J. CELL BIOL.* 110:1041-48; Jensen et al. (1989), *BIOCHEM. ARCHIVES* 5:171-176; Moestrup and Gliemann (1989), *J.*

*BIOL. CHEM.* 264:15574-77; Marynen et al. (1984), *J. BIOL. CHEM.* 259:7075-

- 5 79). The sequence of the human receptor revealed that it is a low density lipoprotein receptor-related protein that binds several other ligands in addition to  $\alpha_2$ M, including lactoferrin, *Pseudomonas* exotoxin A, lipoprotein lipase, apoprotein E-enriched lipoproteins, and urokinase- and tissue type-plasminogen activator/plasminogen activator inhibitor-1 complexes. A 39 kDa receptor  
10 associated protein (RAP) (Williams et al. (1992), *J. BIOL. CHEM.* 267:9035-40) is capable of competing with all of these ligands, including  $\alpha_2$ M, although none of the ligands themselves compete with  $\alpha_2$ M. It is this receptor that is believed to mediate endocytosis.

- 15 Thus, the other receptor is believed to mediate signalling. Several lines of evidence point to a separate receptor for signalling. First, binding of  $\alpha_2$ M to its receptor elicits intracellular signalling cascades, including an increase in intracellular  $Ca^{2+}$  concentration; an increase in cyclic AMP; generation of inositol triphosphates and tetraphosphates (Uhing et al. (1991), *BIOCHIM. BIOPHYS.*  
20 *ACTA* 1093:115-120; Misra et al. (1993), *BIOCHEM. J.* 290:885-891); and activation of protein kinase C (data contained herein). These events are characteristic of G-protein coupled receptor signalling cascades. Consistent with this hypothesis is the observation that nonhydrolyzable GTP analogs and inhibitors can block the  $\alpha_2$ M-R signal transduction mechanism. However, a typical G-  
25 protein coupled receptor contains seven transmembrane spanning domains, whereas the putative endocytic receptor contains only one.

Second, cis-DDP treatment of f- $\alpha_2$ M or the 40 kDa fragment of  $\alpha_2$ M reduces the binding affinity to macrophages. For example, the  $K_d$  for binding of f- $\alpha_2$ M to

- 30 murine macrophages increases from 0.5 nM to 11.0 nM upon treatment with cis-DDP; the  $K_d$  for binding of the 40 kDa RBD increases from 5 nM to 50 nM.

(The  $K_d$  of the 20 kDa RBD is not affected by treatment with cis-DDP.)

Nevertheless, treatment of f- $\alpha_2$ M and the 40 kDa RBD with cis-DDP has no effect on signalling ability. These independent effects, increase in  $K_d$  without changing signalling, are more likely to result from the actions of two binding receptors, one

- 5 of which may be present in higher numbers than the other.

Third, the inhibitor of ligand binding to the endocytic receptor, receptor associated protein (RAP), abolishes f- $\alpha_2$ M binding to the endocytic receptor, but failed either to elicit a calcium signal or to antagonize intracellular signals elicited by either f-

- 10  $\alpha_2$ M or RBF.

As noted above, binding to the signalling receptor can occur independent of binding to the endocytic receptor. Thus, the invention provides for specifically targeting the signalling receptor and activating macrophages or other APCs. In

- 15 addition to the signalling cascade events noted above,  $\alpha_2$ M-mediated signalling can include one or more of the following: activation of protein kinase C;

phosphorylation of histones; and transport of diacylglycerol (DAG) analogs to the cell membrane. Thus, the invention advantageously provides a method to affect the balance between the endocytic  $\alpha_2$ M receptor and the  $\alpha_2$ M signalling receptor

- 20 activities, which may be important in regulating cell growth and differentiation, particularly in immune cells.

Thus, as can be readily appreciated, one of the discoveries of the invention is that  $\alpha_2$ M, or a fragment thereof, that is capable of binding to a receptor, is useful for

- 25 immunomodulation whether or not the  $\alpha_2$ M forms a complex with an antigen.

Thus, it is a particular advantage of the invention to provide, in one aspect,  $\alpha_2$ M, or fragments thereof, free of any other protein or other component complexed therewith.

- 30 In one aspect of the invention, the 20 kDa RBF targets the signalling receptor. In another aspect of the invention, cis-DDP or oxidized f- $\alpha_2$ M or the 40 kDa RBD

- specifically targets the signalling receptor, as treatment with cis-DDP or oxidation appears to significantly diminish binding to the endocytic receptor. Oxidation can be effected by treatment with an oxidant, such as but not limited to peroxide, in particular hydrogen peroxide, hypochlorous acid, or chloramines, or with a free radical such as an oxygen radical. An advantage of oxidation is that the reagents are consumed in the reaction, and further purification is minimal or unnecessary.
- 5 cis-DDP, on the other hand, is toxic, and therefore any free cis-DDP remaining following the reaction with  $\alpha_2M$  or fragments thereof must be removed.
- 10 In addition to using specific fragments or chemically treated  $\alpha_2M$ , specific targeting of the endocytic or signalling receptor can be accomplished by various strategies. For example, the relative cellular expression of one or the other receptor can be altered, *e.g.*, using anti-sense technology or specific cytokines, such as  $\gamma$ -interferon or other hormones that downregulate the endocytic receptor.
- 15 In another embodiment, competitors for the endocytic receptor, such as but not limited to RAP, can be provided.
- Specifically targeting the signalling receptor, without binding to the endocytic receptor, can promote signalling for a longer time period, and provide for a longer 20 acting complex, as the RBF and cis-DDP treated or oxidized f- $\alpha_2M$  or RBD are cleared more slowly. A further advantage of the RBF is that its small size facilitates production, purification and administration.
- Furthermore, it has been found that the RBF is as potent or more potent than f- 25  $\alpha_2M$  for inducing signalling. For example, 40 nM of the 20 kDa RBF was found to be equivalent to 200 nM  $\alpha_2M$  in a signal cascade assay. Further, 2  $\mu$ m doses of RBF stimulated such massive calcium fluxes that it became cytotoxic; an effect not seen with  $\mu$ m doses of  $\alpha_2M$ .
- 30 The invention further provides for immunostimulation without specifically targeting an antigen by providing  $\alpha_2M$  or fragments thereof that are targeted

specifically to the signalling receptor and not to the endocytic receptor. As pointed out in an Example, *infra*, f- $\alpha_2$ M that has not been cis-DDP treated or oxidized can nevertheless enhance an immune response. According to the invention,  $\alpha_2$ M or a fragment thereof targeted specifically to the signalling

- 5 receptor can be administered to a subject in whom immunomodulation, in particular, immunostimulation, is desired. Preferably, such administration is concomitant with or in an admixture with an antigen or vaccine.

In yet a further embodiment, the signalling receptor targeted  $\alpha_2$ M or fragment

- 10 thereof of the invention, whether conjugated with antigen or used for its immunostimulatory effects, can be used in conjunction or synergy with additional immunomodulatory agents, such as but not limited to lymphokines (*e.g.*, interleukin (IL)-1, IL-2, IL-3, IL-4, IL-6, etc.), cytokines (*e.g.*, macrophage inflammatory proteins, interferons, tumor necrosis factor, colony stimulating factors, etc.), growth factors, and the like, to achieve greater immunomodulation, and particularly immunostimulation. Alternatively, very high doses of the RBF can be used to selectively injure immune cells bearing the signalling  $\alpha_2$ M receptor.

Suitable antigens intended for the practice of the present invention may possess

- 20 nucleophilic groups, as  $\alpha_2$ M exhibits a particular facility for the covalent attachment of nucleophilic moieties. They may also include other molecules for which a chemical crosslinker is available that allows attachment to engineered carboxyl terminus  $\alpha$ -macroglobulin derivatives. Exemplary antigens may be selected from peptides, proteins, cytokines, growth factors, hormones, enzymes, 25 toxins, nucleic acids, in particular, anti-sense RNA, as well as other drugs, oligonucleotides and carbohydrates.

Likewise, the invention includes multiple active fragments of  $\alpha_2$ M associated in tandem relationship, such as carboxyl terminal moieties with cross-linking and/or

- 30 receptor recognition sites associated therewith, disposed in direct connection with each other. In such event, multiple antigens both identical and diverse may be

associated with such corresponding multiple fragments to facilitate a polyvalent vaccine delivery construct. Alternatively, such polyvalent construct may be achieved with a single fragment to which is attached an appropriate polyvalent moiety. Both concepts and constructs are included within the scope of the present  
5 invention.

As indicated earlier, the present invention is predicated on the discovery that the formation of the complex or construct between the antigen and  $\alpha_2M$  or its active fragments results in significant improvement in antigen presentation *in vitro* and  
10 more importantly, a dramatic increase in immune activity as measured by the development of antibodies to the antigen stimulus *in vivo*. This significant increase in activity is one aspect of the invention, the other being the ability of the complex or constructs of the present invention to be presented without use or inclusion of an adjuvant. As the data presented later on herein shows,  
15 improvement in immune response is achieved over like formulations with adjuvant. The ability to delete adjuvant from the formulations prepared in the present invention represents a further efficiency and likewise eliminates the potential for deleterious reactions and delays in uptake that have been experienced with adjuvant-containing formulations.

20

The present invention further extends to methods for the preparation of antibodies to such antigens, including where desired, the preparation of monoclonal and chimeric antibodies based upon those raised against the complexes and/or constructs of the present invention, as well as "primed" lymphocytes specific for  
25 the antigens. Likewise, the present invention can be used as a means for stimulating antigenicity and immunocompetence in instances where the particular antigen has previously failed to elicit immunologically or therapeutically significant arousal and activity in the host.

30 The present invention is primarily directed to the administration of antigens recognized by the macrophage in view of the existence on the macrophage of

receptors for  $\alpha_2$ -macroglobulin. However, other APCs may possess receptors for  $\alpha_2M$  and the present invention is accordingly intended to extend to the presentation of antigen to these other APCs.

- 5 By combining the antigen with  $\alpha_2$ -macroglobulin or an active fragment thereof to form the complex or construct of the invention and using the complex or construct as the immunogen, a "modified immune response" can be achieved. This means that, e.g., the immunogen can be used to raise antibodies which are specific to epitopes either weakly or not previously recognized. Additionally, the modified immune response may involve non-antibody immune system components, e.g., T-lymphocytes, which may recognize an epitope not previously presented or recognized. Hence, the "modified immune response" is largely directed to the previously weakly or unrecognized epitope on the antigen treated, or epitopes requiring adjuvant or use of large amounts of antigen, all as described herein.
- 10
- 15

The preferred embodiments of the invention utilize the complex or construct as the immunogen, and seek to raise or react said complex or construct with antibodies which also recognize the same or a different epitope which is present on the molecule. In this aspect of the invention, the so-called modified immune response therefore involves the generation of antibodies which are not otherwise efficiently formed or observed *in vitro* or *in vivo*. It may also involve generation of antibodies or stimulation of lymphocytes that would not otherwise occur in the absence of noxious adjuvants not approved for human usage. Preferably, and advantageously, such antibodies can be generated by immunization in the absence of adjuvant. For example, the immunogen can be used to inoculate a mammal to raise antibodies to the newly recognizable epitope, and to produce antiserum or vaccine preparations, and the like.

Likewise, antibody molecules can be cleaved to form antibody fragments, which can be recombined *in vitro* to form chimeric antibodies which recognize or bind to newly recognizable epitopes on the antigen. Hence, the "modified immune

"response" is not limited to a conventional immune response, or to increases or decreases in the extent or severity thereof.

As stated earlier, both positive and negative regulation of the antigenicity of epitopes can be achieved. For example, by rendering epitopes recognized, or recognizable, antibodies can be raised to recognize and bind to the antigen. Enhanced antigenicity and the ability to raise antibodies to otherwise weak, scarce or ineffective epitopes finds great utility not only, for example, in vaccine applications in animals, including humans, but also in producing antibodies which can be used as reagents for, among other uses, binding, identifying, characterizing and precipitating epitopes and antigens, such as the production of antibodies against scarce antigens for research purposes.

Also, immunodominance of particular epitopes on a molecule may be modified. Certain antigens containing more than one epitope have characteristic immune responses based upon the dominance of one epitope over the other(s). This aspect of the invention enhances the recognition of the subordinate epitope(s) by either preparing and administering a complex or construct of the invention to potentiate the recognition and activation of the subordinate epitope(s), or by preparing and administering a complex or construct bearing an agent that will be recognized by the dominant epitope and suppress the recognition of the same by antigen. In this connection, a multivalent construct could be prepared that bears both the antigen for the subordinate epitope and the inhibitor or down regulator for the immunodominant epitope. This allows other silent or recessive epitope(s) to be expressed.

In another alternative preferred aspect of the invention, the present constructs may be prepared recombinantly. This, again, can be undertaken by the incorporation of the antigen within the carboxyl terminal fragment, inserting the resulting construct into an expression vector, transfecting the vector into a host and allowing the host to express the construct with the antigen added thereto.

An expression vector may be prepared that codes for expression of a mutein of the molecule of interest, which vector may be transfected into a host cell, such that the cell is caused to express the mutein. The mutein may comprise the original amino acid sequence of the construct, conserved variants thereof or portions

- 5 thereof, substituted with or having inserted therein codons for expression of the antigen in the primary sequence of the mutein, in the instance where the antigen is capable of such replication.

- A further embodiment may for example, take advantage of APC receptor proteins  
10 which recognize and bind to polypeptide molecules present on the antigen or in the complex or construct of the invention.

- Antigen uptake by the APCs can occur via nonspecific mechanisms, and may be followed by display of the antigen in association with MHC on the cell surface.  
15

- Once antigen is internalized by APCs, partial proteolytic degradation occurs in a prelysozomal endosome, and processed peptide fragments of the antigen become associated with MHC molecules. However, while partial proteolytic degradation of antigen may be essential in order to generate appropriate MHC and T-cell  
20 binding to the peptide fragments thereof, excessive degradation of antigen has been found to be detrimental to the eventual immune response. Inhibition of proteolysis which is not essential for the processing of a specific antigen has been shown to enhance processing and presentation, suggesting that the interference with inappropriate proteolysis actually enhances antigen presentation.  
25

- These two processes, targeting antigen to the surface of APCs, and interfering with nonessential antigen proteolysis, can be used herein to enhance antigen processing and presentation. For example, by preparing the antigen in the complex or construct of the invention, and then combining the complex or  
30 construct with APCs, different fragmentation and presentation patterns may result.

Likewise by fragmenting the antigen prior to preparation of the complex or construct, fragmentation and presentation patterns may be modifiable.

- In a specific embodiment, protection of peptides complexed with  $\alpha_2M$  from  
5 degradation, in particular proteolysis, can be demonstrated. For example, a peptide having the sequence:



- can be prepared synthetically. In such a peptide, Lys<sub>1</sub> can provide a side for  
10 crosslinking to Glx of the  $\alpha_2M$  thioester; Cys<sub>5</sub> can provide a site for labelling with [<sup>14</sup>C]-uracilic acid; Glu<sub>8</sub> provides a cleavage site for V8 protease [MW 28 kilo-Daltons (kDa)]; and Tyr<sub>13</sub> can provide a site for <sup>125</sup>I-labelling. A complex of this peptide and  $\alpha_2M$  can be formed by PPE activation of  $\alpha_2M$  in the presence of  
15 peptide. This complex can then be treated with V8 protease, and the amount of <sup>125</sup>I released from the complex measured to determine whether the peptide is protected from V8 protease. The <sup>14</sup>C provides a control for covalent crosslinking to  $\alpha_2M$ . If this model peptide is protected once bound to  $\alpha_2M$ , it follows that  $\alpha_2M$  can protect peptides in general.
- 20 The biological processes within the APCs can be controlled to enable one to qualitate or quantitate the binding of the complex or construct. For example, the incubation time and temperature can be adjusted to achieve complete internalization by APCs, complete binding of the complex or construct, and like parameters. By maintaining APCs and in combination at an appropriate  
25 temperature, e.g., about 4°C, for an appropriate time period, e.g., about one hour, binding of the complex or construct to APC cell surfaces can be quantitated, since internalization can be effectively decreased or shut down. Alternatively, by increasing the APC/complex or construct incubation temperature and/or time period, e.g., up to about 37°C for about one hour, internalization can be  
30 evaluated.

The antibodies described herein are typically those which recognize the epitopes on the antigens which are made recognizable, enhanced or suppressed as described above. By injecting this type of antigen into a mammal, such as through a hyperimmunization protocol, modulated antibody responses or CTL responses to 5 the epitopes can be achieved.

The antibodies which are disclosed herein may be polyclonal, monoclonal or chimeric antibodies, and may be raised to recognize the desired epitope and used in a variety of diagnostic, therapeutic and research applications. For example, the 10 antibodies can be used to screen expression libraries to ultimately obtain the gene that encodes proteins bearing the epitope evaluated. Further, antibodies that recognize the antigen presented can be employed or measured in intact animals to better elucidate the biological role that the protein plays, or to assess the state of immune response or immunologic memory more effectively.

15

Polyclonal, monoclonal and chimeric antibodies to the antigen can be prepared by well known techniques after immunization with a complex according to the invention, such as the hyperimmunization protocol, or the hybridoma technique, utilizing, for example, fused mouse spleen lymphocytes and myeloma cells.

20 Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Likewise, chimeric antibody molecules can be produced using an appropriate transfection and hybridoma protocol. In an analogous fashion, immortalized epitope-specific T-lymphocyte lines can also be 25 developed.

The present invention also includes the immunogens which are produced and used as described herein in form. Thus, the preferred immunogen is an antigen prepared in a complex or construct of the invention, which has at least one 30 epitope. The immunogen has modified antigenicity due to the presence of, reaction with or linkage to the  $\alpha_2M$  molecule or construct. The immunogen

induces the formation or proliferation of T-cells or antibodies which recognize the protein in its modified form or in its non-modified form.

The present invention provides for formation of covalent complexes of antigen and  
5  $\alpha_2M$  or C-terminal fragments thereof. Such covalent complexes are  
advantageously formed by reaction of a nucleophile on the antigen, such as an  
amine functional group (e.g., the  $\epsilon$ -amino group of lysine, or a  $\alpha$ -amino group of  
a peptide) or hydroxyl, with the thioester of  $\alpha_2M$  during activation of  $\alpha_2M$  with  
protease. However, the invention contemplates covalently coupling an antigen to  
10  $\alpha_2M$  or fragments thereof using any bifunctional crosslinking agent known in the  
art.

In a preferred embodiment, the antigen used in an immunogenic complex of the  
invention is a synthetic HIV peptide, e.g., as described in Hart et al. ((1991),  
15 *PROC. NATL. ACAD. SCI. U.S.A.* 88:9448-52). Such synthetic peptides combine  
neutralizing B-cell sites from the third variable region (V3) of the HIV envelope  
peptide gp120, with the gp120 T-helper epitope T-1. Several of these synthetic  
peptides, designated T1-SP10, have been demonstrated to elicit high-titered  
neutralizing antibodies and T-cell responses in mice, goats, and rhesus monkeys,  
20 when administered in incomplete Freund's adjuvant (*see* Hart et al., *supra*). For  
example, the peptide T1-SP10MN(A) (MW 4771), which has the following amino  
acid sequence: KQIINMWQEVGKAMYACTRPYNKRKRIHIGPGRAYTTK  
(SEQ ID NO:2), can be complexed with  $\alpha_2M$ , or a C-terminal fragment thereof,  
either via the endogenous  $\alpha_2M$  thioester or using a bifunctional crosslinker, such  
25 as the homo-bifunctional sulphydryl reactive crosslinker bis-moleimidohexane  
(BMH) to methamine (ma)-treated  $\alpha_2M$ , e.g., by reacting an excess of peptide  
(100-fold, for example) and crosslinker (10-fold, for example) with  $\alpha_2M$ -ma  
overnight at 4°C.

The present invention also contemplates diagnostic and therapeutic applications for these agents. Accordingly, the antigens or antibodies thereto may be prepared for use in a variety of these methods.

- 5 Any of these agents may be labeled or unlabeled as appropriate. Typically the labelled component is the antibody, but it is possible to label the antigen or the  $\alpha_2M$  component, MHC or APCs as well.

Thus, both receptors and the binding partners which recognize the antigen  
10 presented are used in connection with the various techniques described herein. For example, a radioimmunoassay may be conducted, using for example, an antibody or ligand, that may either be labeled or unlabeled. Labelling may be accomplished, e.g., by radioactive addition, reduction with sodium borohydride or radioiodination.

15

Labels most commonly employed are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light and others.

- Suitable radioactive elements may be selected for the group consisting of  $^3H$ ,  $^{14}C$ ,  
20  $^{32}P$ ,  $^{33}P$ ,  $^{35}S$ ,  $^{36}Cl$ ,  $^{51}Cr$ ,  $^{57}Co$ ,  $^{58}Co$ ,  $^{59}Fe$ ,  $^{90}Y$ ,  $^{125}I$ ,  $^{131}I$ , and  $^{186}Re$ . In the instance where a radioactive label, such is presented with one of the above isotopes is used, known currently available counting procedures may be utilized.

- In the instance where the label is an enzyme, detection may be accomplished by  
25 any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, thermometric, amperometric or gasometric techniques known in the art. The enzyme may be conjugated to the antigens or antibodies, their binding partners or carrier molecules, by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Also, and in a  
30 preferred embodiment of the present invention, the enzymes themselves may be

modified into advanced glycosylation endproducts by reaction with sugars as set forth herein.

Many enzymes which can be used in these procedures are known and can be

- 5 utilized. The preferred are peroxidase,  $\beta$ -glucuronidase,  $\beta$ -D-glucosidase,  $\beta$ -D-galactosidase, urease, glucose oxidase plus peroxidase, hexokinase plus GPDase, RNase, glucose oxidase plus alkaline phosphatase, NAD oxidoreductase plus luciferase, phosphofructokinase plus phosphoenolpyruvate carboxylase, aspartate aminotransferase plus phosphoenol pyruvate decarboxylase, and alkaline 10 phosphatase. U.S. Patent Nos. 3,654,090; 3,8,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternative labeling material and methods.

A number of fluorescent materials are known and can be utilized as labels. These

- 15 include, for example, fluorescein, rhodamine and auramine. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

In an immunoassay, a control quantity of a binding partner to a antigen

- 20 complex/construct may be prepared and optionally labeled, such as with an enzyme, a compound that fluoresces and/or a radioactive element, and may then be introduced into a tissue or fluid sample taken from a mammal in order to assess, e.g., the amount of antigen present therein. After the labeled material or its binding partner(s) has had an opportunity to react with the sample, the resulting 25 complex may be examined by known techniques, which vary according to the nature of the label attached. In this manner the antigen receptor, the activity and effect of MHC, or the epitope recognized by the antibody can be evaluated.

In each instance, the antigen complex/construct forms complexes with one or more

- 30 binding partners and one member may be labeled with a detectable label. The fact

that a complex has formed and, if desired, the amount thereof, can be determined by the detection methods described herein.

One preferred diagnostic method included herein involves the determination of T-

- 5 lymphocyte levels, function or activity in a sample taken from a mammal. The immunogen comprising the present complex/construct is incubated with APCs, after which T-lymphocytes taken from the mammal are added. The level, function or activity of the T-lymphocytes taken from the mammal can then be compared to a standard.

10

In one preferred embodiment, the APCs can be associated with a solid support.

In another preferred embodiment, the immunogen is combined with APCs at a temperature which is effective to cause binding between the APCs and the

- 15 immunogen. This can be accomplished without allowing substantial internalization by the APCs. In this manner, antigen binding to said APCs can be evaluated. Also, by increasing the temperature, APC internalization of antigen and subsequent cell metabolic processes can be evaluated.

- 20 Therapeutic treatments and diagnostic methods can be performed using any or all of the various components and processes described herein. For example, for the diagnosis or treatment of cancer or infection, an isolated protein can be derived from the tumor, abnormal cells or infectious organism, and this protein can be used as an antigen and prepared in a complex or construct. Antibodies to this

- 25 protein can be elicited using the methods for enhanced antigen presentation disclosed herein and used to identify, characterize, bind, inhibit or inactivate, as desired, previously unknown or ineffective epitopes on the tumor, abnormal cell, bacterial or viral protein. This information, in turn, is useful for developing drugs which combat such afflictions, such as agonists, antagonists and the like.

30

Likewise, the antibodies described above can be raised to have direct diagnostic or therapeutic utility, particularly in oncologic, autoimmune and infectious disease treatments.

- 5 A preferred use for the antigen complex/constructs described herein is in the form of a vaccine which can be used to immunize mammalian patients in need of such treatment. By administering to such patient an effective amount of the immunogen, antibodies can be raised to the particular immunogen and immunogen-specific lymphocytes can be primed and activated, which are effective
- 10 for treating disease or preventing the development or spread thereof. In a specific embodiment, the invention provides a vaccine against HIV.

The preferred non-cellular components which recognize antigen and which are used to characterize epitopes presented in accordance with the invention include  
15 the antibodies raised to an antigen which are not normally elicited in the absence of the methods described herein. Also, as noted above, the most preferred antibodies are raised to antigen in the complex/construct, but recognize the non-modified molecule.

- 20 The general procedures set forth above are illustrated in the following examples. All of the protocols disclosed herein may be applied to the qualitative and quantitative determination of epitopes activated by the processes set forth herein.

#### EXAMPLE 1

25

We proposed that this thiolester-mediated covalent bond formation may reflect an important functional role. Using T-hybridoma cells specific for hen egg lysozyme (HEL), in conjunction with various HEL and/or  $\alpha_2M$  derivatives, we probed the effect of HEL- $\alpha_2M$  complex formation on Ag uptake and processing by murine  
30 macrophages. The results indicated that  $\alpha_2M$  was capable of mediating

receptor-facilitated Ag delivery to macrophages, enhancing presentation of the Ag to specific T-cells. The details of the studies follow below.

### METHODS

- 5 *Materials.* Hen egg white lysozyme (HEL) was purchased from Boehringer Mannheim (Indianapolis, IN).  $\alpha_2M$  was purified as previously described (22, 23), except that buffers made from pyrogen-free sterile water (Abbott Laboratories, Chicago, IL) were used for extensive washing and elution. Fractions were analyzed by pore limit gel electrophoresis in a tris/boric acid/EDTA (TBE) buffered system [(24), modified from (25)], and those fractions containing any trace amounts of "fast" form were discarded, resulting in material that was >98% native, as determined by DTNB titration (20, 24). Porcine pancreatic elastase (PPE) of the higher purity grade was purchased from Sigma (St. Louis, MO). Human neutrophil elastase was the gift of Drs. James Travis and Wieslaw Watorek, University of Georgia, Athens, GA. Carrier-free Na<sup>125</sup>I and [methyl-<sup>3</sup>H]-thymidine were obtained from New England Nuclear (Boston, MA).
- 10
- 15

*Radiolabelling.* HEL and PPE were radiolabelled using an Iodo-Bead™ (Pierce, Rockford, IL), employing conditions recommended by the manufacturer, and desalted on PD-10 columns (Pharmacia, Piscataway, NJ). All <sup>125</sup>I-labelled materials were counted in a LKB-Wallac Clinigamma counter 1272 (Piscataway, NJ).

- 20
  - 25
  - 30
- The concentrations of proteins in solution were determined in a Shimadzu UV 160U spectrophotometer (Columbia, MD), using the following constants:  $A^{1\% / 1cm}_{280nm} = 8.93$  for  $\alpha_2M$  (26),  $A^{1\% / 1cm}_{280nm} = 26.5$  for HEL (27), and  $A^{1\% / 1cm}_{280nm} = 10$  as determined for  $\alpha_2M$ -HEL complexes using amino acid analysis (28).
- Characterization of HEL incorporation.* The binding of radiolabelled HEL to  $\alpha_2M$  was analyzed by systematically varying conditions as previously described for

insulin (20). Incubation products were analyzed by gel electrophoresis and autoradiography using native 4-20% TBE gels (24) and denaturing 5-15% polyacrylamide gradient gels in an ammediol-buffered SDS system (29). Radioactive bands were excised and counted after autoradiography.

5

*Ag Preparations.* HEL- $\alpha_2$ M-PPE complexes were prepared in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HEPES buffer) by incubating 1.4 mM  $\alpha_2$ M with a two-fold molar excess of PPE for 15 min at room temperature in the presence of a 100-fold molar excess of HEL.  $\alpha_2$ M-PPE complexes were formed in similar

- 10 incubations omitting the HEL. After addition of 3,4-dichloroisocoumarin (100 mM) to inhibit bound and unbound proteinase, complexes were separated from unbound HEL and PPE by gel filtration utilizing a Sephadryl® S-300 HR column (Pharmacia/LKB, Piscataway, NJ), as previously described (20). Proteinases were active-site standardized as previously described (30).
- 15  $\alpha_2$ M-methylamine was formed as previously described (31). All buffers used were prepared with pyrogen-free water and filtered through 0.22 mm filters.

Solutions of HEL and of the complexes were passed through a 2 ml Detoxi-Gel™ column (Pierce, Rockford, IL) several times. Final endotoxin levels in the

- 20 presentation assays were <0.1 ng endotoxin/ml, as assessed by Pyrotell *Limulus* amebocyte lysate clotting times (Associates of Cape Cod, Inc., Woods Hole, MA) using a THERMOmax™ microplate reader (Molecular Devices, Menlo Park, CA) according to manufacturer's recommendations. Native  $\alpha_2$ M was inhibitory in this assay, probably due to its proteinase inhibitory properties; therefore, those samples
- 25 were heat inactivated prior to assay. Ag preparations were concentrated in sterilized Centriprep™-30 concentrators (Amicon, Beverly, MA), and filtered through 0.22 mm filters (Millipore, Bedford, MA). Ag dilutions were prepared immediately before use in 96-well polypropylene plates (Costar, Cambridge, MA).

- 30 *Cell Lines.* Peptone-induced peritoneal macrophages were harvested from female CBA/J mice (Jackson Laboratories, Bar Harbor, ME) and allowed to adhere to

96-well tissue culture plates (Costar, Cambridge, MA) for 2 h before use (32).

The 3A9 T-cell hybridoma line specific for residues 52-61 of HEL (33, 34), and the IL-2-dependent CTL line (CTLL-2) (35) were kindly provided by Peter Cresswell, Yale University, New Haven, CT. Both cell lines were grown in

- 5 RPMI 1640 (GIBCO, Gaithersburg, MD), supplemented with 10% heat-inactivated newborn calf serum (Hyclone Laboratories, Inc., Logan, UT), 1% (v/v) l-glutamine (200 mM, GIBCO), and 0.5% (v/v) gentamicin solution (10 mg/ml, GIBCO). The CTLL cell media was further supplemented with 20% rat spleen cell Con A supernatant, prepared as previously described (36).

10

*Uptake studies.* Internalization of  $^{125}\text{I}$ -HEL- $\alpha_2\text{M}$ -PPE complexes and of free  $^{125}\text{I}$ -HEL were studied at 37°C using monolayers of macrophages plated on Dynatech (Chantilly, VA) Removacell® plates. The respective Ag were diluted in HEPES buffer containing 10 mg/ml BSA to obtain the desired concentrations of

- 15 radiolabelled Ag and of unlabelled competitors. This was added to macrophages in RPMI 1640. After specified time intervals, the incubation was terminated by aspiration, and the cells underwent extensive ice-cold washes. Wells were allowed to air dry before counting in a gamma counter.

- 20 *Ag presentation assay.* A well-characterized *in vitro* Ag presentation system was used (37, 33, 34). Macrophages ( $10^5$ ) pulsed with dilutions of various HEL- and/or  $\alpha_2\text{M}$ -derivatives in serum-free media were tested for their ability to induce IL-2 secretion by 3A9 T-hybridoma cells ( $10^5$ ). Extensive washing with RPMI 1640 over a 30 min period was employed to remove uninternalized Ag from the
- 25 96-well plates before addition of 3A9 cells in serum-containing media. After 24 h of incubation at 37°C, 100 ml of culture supernatant was removed and frozen at -70°C to lyse any transferred cells. In some experiments, Ag, 3A9 cells, and macrophages were coincubated for 24 h followed by harvesting of the supernatant. The 3A9 T-hybridoma response was quantitated by measuring the amount of IL-2 released into the supernatants using [ $^3\text{H}$ ]-thymidine incorporation in the CTLL-2 line (36). A Skatron automated combi-harvester (Sterling, VA) was used,

followed by counting in a Packard Minaxi $\beta$  Tri-Carb 4000 liquid scintillation counter (San Fernando, CA). The values from control incubations that lacked Ag, which were < 3.5% of the total, were defined as the baseline and subtracted.

## 5 RESULTS

- Characterization of HEL binding to  $\alpha_2M$ .* The interaction between HEL and  $\alpha_2M$  was characterized, and the predominant mechanism of binding was found to parallel that of insulin (20). Covalent binding of HEL occurred only if HEL was present during proteinase-induced  $\alpha_2M$  conformational change (Figure 1. Panel B, 10 lanes b). Native gels demonstrated that there was more adherence to f- $\alpha_2M$  than seen with insulin (Panel D, lane c), probably due to the basic nature of HEL, but total binding was greatest upon coincubation of HEL,  $\alpha_2M$ , and proteinase (Panel D, lane b).
- 15 The Coomassie-stained SDS gel (Panel A) reveals a typical fragmentation pattern for  $\alpha$ -macroglobulins (38). Under nonreducing conditions, the 720 kDa protein dissociates into disulfide-linked dimers (360 kDa). Under reducing conditions,  $\alpha_2M$  migrates as its constituent 180 kDa subunits. Denaturation and boiling promotes autolytic cleavage at the thioesters, resulting in the characteristic 120 kDa and 60 kDa bands (39). Proteinase-treated  $\alpha_2M$  migrates as a doublet (~90 kDa) (40) when examined by reducing SDS-PAGE (Panel A, lane c); the binding of HEL to the thioester-containing fragment resulted in the appearance of a new band whose position is marked by the arrow (Panel A, lane b). All of the bound  $^{125}I$ -HEL migrated at this position. Some uncleaved  $\alpha_2M$  subunits (180 kDa), and 20 a small amount of material that underwent autolytic cleavage (120 kDa and 60 kDa), can be seen in the reduced proteinase-treated samples (Panel A, lanes b & c) because a subsaturating amount of PPE relative to  $\alpha_2M$  was used. The band just below the intact subunits in lanes b & c represent PPE-bound to the thioester-containing fragment (7). All of the  $\alpha_2M$ -PPE and HEL- $\alpha_2M$ -PPE complexes used in the cellular assays were formed using saturating levels of PPE, 25 and were repurified by gel filtration. When analyzed by reducing SDS-PAGE,

there were no extraneous bands other than the expected doublet or triplet, respectively.

- To further verify that covalent HEL incorporation into  $\alpha_2M$  reflected the general mechanism of nucleophilic displacement at the activated thiolester, an  $\alpha$ -effect nucleophile  $\beta$ -aminopropionitrile, which has previously been shown to compete for incorporation into the Glx of the activated  $\alpha_2M$  thiolester, was employed (7, 20). This nucleophile effectively competed away all covalent binding (Figure 2A), confirming the mechanism. In contrast to proteinase binding to  $\alpha_2M$ , where  $\beta$ -aminopropionitrile competes away covalent binding, but does not affect noncovalent "trapping" (not shown) or "inhibition" (7), the loss of covalent HEL binding resulted in a loss of > 85% of total binding. This may indicate that HEL, being smaller than most proteinases, is not effectively trapped and requires covalent bond formation to remain associated with  $\alpha_2M$ .
- In order to fully characterize the composition of HEL- $\alpha_2M$ -PPE complexes for use in the cellular assays, dose response studies were conducted to determine the stoichiometry of the bound components. Under saturating conditions, about one mole HEL (Figure 2B) and one mole of PPE (not shown) were bound to each mole of  $\alpha_2M$ . About 85% of HEL binding was covalent and resistant to reduction, again supporting the existence of a  $\gamma$ -glutamyl linkage. The use of human neutrophil elastase as the activating proteinase also resulted in a maximum of one mole HEL bound per mole  $\alpha_2M$ , but in this case, binding was 100% covalent. These results differ from those of insulin, which demonstrated a maximum of 3.7 moles bound per mole  $\alpha_2M$  (20), probably reflecting the greater size of HEL relative to insulin. Proteinase trapping, which was usually ~ 1.5:1 (mol PPE/mol  $\alpha_2M$ ) in the absence of HEL, decreased to 1:1 as HEL binding increased, lending further evidence that HEL was inside the trap.
- Internalization of HEL derivatives by macrophages.* After characterizing the composition of the various complexes, their relative rates of uptake by macrophage

monolayers were compared. In these uptake assays, as well as in the subsequent Ag presentation assays, concentrations of the HEL- $\alpha_2$ M-PPE complexes were adjusted relative to that of free HEL so that the corresponding incubations contained the same molar amount of HEL, whether bound or free. Because f- $\alpha_2$ M tended to precipitate during concentration, and because the calcium and magnesium requirements of  $\alpha_2$ M receptor-mediated endocytosis (9, 41) necessitated diluting Ag preparations into media, concentrations of HEL- $\alpha_2$ M-PPE greater than about 1 mM could not be examined. Small primary amines, such as methylamine, can directly substitute into the thiolester, resulting in f- $\alpha_2$ M complexes that lack proteinase (42). Methylamine-treated  $\alpha_2$ M interacts with the receptor in a manner indistinguishable from  $\alpha_2$ M-proteinase complexes (23). Thus, it can be used either as a specific competitor for  $\alpha_2$ M-proteinase complexes or as a method to investigate the effects of  $\alpha_2$ M receptor-binding in a context free of proteinases.

15 Specific uptake of the  $^{125}$ I-HEL- $\alpha_2$ M-PPE complex proceeded rapidly in a manner consistent with published literature on f- $\alpha_2$ M uptake (15, 41, 43) (Figure 3). This uptake was sensitive to competition by  $\alpha_2$ M-methylamine, but not by unlabelled HEL (Figure 4A). The specific uptake of free  $^{125}$ I-HEL was much less, and

20 displayed a 50-90 min time lag before values above the baseline were detected (Figure 3). It is not clear what mechanism accounts for the uptake of free  $^{125}$ I-HEL since neither unlabelled HEL nor  $\alpha_2$ M were able to compete with it effectively (Figure 4A). Nevertheless, these studies demonstrate that  $\alpha_2$ M is capable of delivering Ag into macrophages effectively, resulting in a higher level

25 of internalization than observed with free Ag.

*Presentation to HEL-specific T-hybridoma clones.* An Ag presentation assay that measured the activation of HEL-specific T-hybridoma cells was then employed to study the effects of enhanced internalization upon Ag processing. Complexing

30 HEL to  $\alpha_2$ M lowered the threshold for achieving detectable T-cell responses by 2.2-2.7 log units, allowing 2 nM amounts to be effectively presented (Figure 5).

By contrast, more than 400 nM was required for the presentation of free HEL. Since the 3A9 T-hybridomas were added after extensive washing to remove uninternalized Ag, the observed enhancement was not due to an effect of the complex on the T-hybridoma. To control for the possibility that f- $\alpha_2$ M was 5 affecting macrophage metabolism directly through receptor binding events, complexes of  $\alpha_2$ M-PPE or of  $\alpha_2$ M-methylamine were added to free HEL, exposing the macrophages to the same molar amounts of f- $\alpha_2$ M and HEL as when the HEL- $\alpha_2$ M-PPE complexes were given. This had no effect on the presentation of free HEL (Figure 5), confirming that the enhancement observed with the HEL- 10  $\alpha_2$ M-PPE complexes required direct attachment of the HEL to  $\alpha_2$ M.

To verify that receptor-mediated events were critical for the enhanced ability of the macrophages to present  $\alpha_2$ M-complexed HEL, competition studies were performed using receptor-recognized f- $\alpha_2$ M or BSA. The presence of f- $\alpha_2$ M as a 15 competitor during the Ag pulse prevented the macrophages from being able to process and present HEL- $\alpha_2$ M-PPE complexes to the T-cells, whereas 100-fold excesses of BSA had no effect (Figure 4B). These results substantiated the hypothesis that receptor-enhanced uptake of Ag was critical for the observed enhancement in processing efficiency.

20 Since receptor-mediated endocytosis may result in more rapid, as well as higher levels of, Ag uptake, macrophages were pulsed with log-dilutions of Ag for varying amounts of time ranging from 15 min to 3 h. The resulting dose response curves for different macrophage-Ag incubation times were analyzed to 25 determine the minimal concentration of Ag required to enable activation of T-hybridomas by the macrophages. These results are summarized in Figure 6. For both free HEL and HEL- $\alpha_2$ M-PPE complexes, the longer the Ag pulse, the lower the concentration of Ag needed during the pulse. At each time point, however, macrophages exposed to HEL- $\alpha_2$ M-PPE complexes required less than 30 1/100 the amount of Ag required by macrophages exposed to free HEL.

After the initial 50-60 min, the difference in presentation efficiency between free HEL and HEL- $\alpha_2$ M-PPE complexes seemed to decrease slightly with time (Figure 6). To investigate this further, a 24 h coincubation of macrophages, Ag, and 3A9 cells in serum-containing media was conducted. The dose-response curve for presentation of HEL (Figure 7) was consistent with previously published studies (33). Again, nM levels of HEL- $\alpha_2$ M-PPE complexes were sufficient for presentation. No IL-2 was secreted during control incubations involving macrophages, T-hybridomas, and  $\alpha_2$ M-PPE or  $\alpha_2$ M-methylamine. If either macrophages or T-hybridomas were omitted from incubations containing HEL, the resulting supernatant was unable to stimulate CTL growth. Even when present for 24 h, free HEL was still 20 times less efficient than  $\alpha_2$ M-complexed HEL in eliciting T-cell activation (Figure 7). This smaller difference, relative to that of used studies, is due to more efficient presentation of HEL when allowed to interact continuously with macrophages and T-hybridomas for 24 h. Processing of HEL- $\alpha_2$ M-PPE complexes had already reached maximum efficiency after a 2-3 h pulse with the macrophages (Figure 6).

#### DISCUSSION

$\alpha_2$ M displays an unique capacity, upon proteolytic activation, for rapidly forming essentially irreversible complexes with proteins possessing dissimilar structures. The inter-related mechanisms of trapping and of covalent crosslinking allow it to complex with a wide variety of proteinases and other proteins, including large proteinases up to 90-110 kDa (5, 12, 44). The capture of nonproteolytic proteins such as insulin is very efficient, occurring readily at physiologic concentrations of reactants (20). The resulting complexes bind the  $\alpha_2$ M receptor and are effectively internalized. These properties have been exploited to deliver enzymes and hormones into cells for experimental purposes (14, 15). The above represents evidence that the immune system may also exploit these properties to enhance the early stages of nonspecific antigen (Ag) processing by macrophages.

When complexed to  $\alpha_2M$ , HEL was processed and presented much more efficiently than when it was unbound (Figures 3,5,6, & 7). Ag-pulsed macrophages were able to present nM amounts of HEL- $\alpha_2M$ -PPE complexes in contrast to the mM amounts necessary for presentation of free HEL, and of free

- 5     HEL in the presence of f- $\alpha_2M$ . Most previous studies of HEL presentation utilized more than 7  $\mu M$  (100  $\mu g/ml$ ) concentrations of Ag (34, 37, 45). The ability of  $\alpha_2M$  to spontaneously incorporate HEL during its activation by proteinases, allowed examination of macrophage Ag processing at much lower concentrations of Ag, as might be found *in vivo*. Receptor-enhanced uptake of
- 10    HEL- $\alpha_2M$ -PPE complexes also appeared to result in more rapid processing. Although 20 nM of uncomplexed HEL could be presented after 24 h of coincubation with macrophages and T-cells, macrophages could present a comparable level of  $\alpha_2M$ -complexed HEL after only a 15 min pulse (Figure 6).
- 15    The results presented here represent the first evidence for a specific effect of  $\alpha_2M$  on the processing of a particular Ag and its presentation to a homogeneous population of T-hybridoma clones. This study extends and is supported by earlier studies using less specific presentation assays. Macrophages pulsed with  $\alpha$ -galactosidase- $\alpha_2M$ -trypsin complexes were 16 times better at stimulating
- 20    proliferation in T-cells compared to those pulsed with free  $\alpha$ -galactosidase (53). In addition, incubating  $\alpha_2M$ -complexed viral proteins with macrophages and spleen-derived cells resulted in increased anti-viral antibody secretion (54). The levels of covalent and noncovalent binding of Ag to  $\alpha_2M$  were not fully characterized, so it is difficult to know whether the compared incubations
- 25    possessed equivalent molar amounts of  $\alpha$ -galactosidase. While the observed effects could have been the result of nonspecific stimulation of the T-cell population, when considered alongside the results presented here, these earlier studies attest to the potential generality of  $\alpha_2M$ 's ability to enhance uptake and processing of Ag by macrophages.

In addition to HEL,  $\alpha_2$ -galactosidase, and viral proteins,  $\alpha_2$ M has been shown to covalently bind proteins as diverse as lysozyme, aprotinin, inactive forms of trypsin (7), insulin (8, 20), luteinizing hormone and possibly streptokinase (unpublished observations). All these appear to interact with  $\alpha_2$ M by nucleophilic

- 5 attack at the thiolester Glx, as was originally described for the covalent attachment of lysine-containing proteinases (7). This is an efficient process since crosslinking occurs during a reactive intermediate state that decays in seconds (8, 20). Growth factors that appear to bind  $\alpha_2$ M include platelet-derived growth factor, transforming growth factor- $\beta$ , IL-1 $\beta$ , IL-6, and basic fibroblast growth factor  
10 (Reviewed in (21)). The association of these different proteins attests to the versatility of the  $\alpha_2$ M "trap."

Besides being noncovalently trapped or forming covalent  $\epsilon$ -lysyl- $\gamma$ -glutamyl amide bonds with  $\alpha_2$ M, proteins may be captured by additional mechanisms. Basic

- 15 proteins can adhere to  $\alpha_2$ M in a manner distinct from trapping (18). About 15% of the total binding seen with HEL was noncovalent, was not lost with  $\beta$ -aminopropionitrile, and could bind to pre-formed  $\alpha_2$ M-PPE, supporting the possibility of adherence to  $\alpha_2$ M or PPE (Figures 1BD & 2AB). Additional forms of covalent bonds are also possible. The free thiols released by thiolester decay  
20 have been suggested as potential sites for thiol-disulfide interchange (19). In addition, we have recently reported evidence supporting the existence of  $\gamma$ -glutamyl ester bonds (20). Both ester and amide linkages are observed in other members of the  $\alpha$ -macroglobulin superfamily, specifically, the thiolester containing complement components C3 and C4 (55), and possibly *limac*, the  
25 horseshoe crab macroglobulin (56). Interestingly, the covalent bonds formed by the thioesters of *limac* allow it to participate in a hemolytic system, but have no relation to its proteinase inhibitory activity (56).

- Just as the thioesters of the complement components mediate their biologic  
30 activity [Reviewed in (57, 58)], the reactions of the conserved  $\alpha$ -macroglobulin thiolester may reflect potential roles of these proteins in immune processes.

Although thiolester incorporation is critical to the proteinase inhibitory role of the monomeric  $\alpha_2$ -macroglobulins (17), the role of the  $\alpha_2$ M thiolester is a mystery (18). It is not needed for proteinase inhibition by tetrameric and most dimeric  $\alpha_2$ -macroglobulins (6, 7, 56, 59, 60), but may perhaps be essential to the ability of

- 5  $\alpha_2$ M to capture smaller proteins. The 14 kDa HEL appears to slip out of the closed trap if covalent binding is abolished by  $\beta$ -aminopropionitrile (Figure 2A). This is consistent with earlier observations that proteins smaller than 20 kDa appear capable of diffusing through the arms of the "sprung" trap (5, 50, 60, 61). Thus, thiolester-mediated covalent linkage may be essential to the efficient capture  
10 of potential Ag.

It is proposed that  $\alpha_2$ M may act to target potential Ag to macrophages within areas of inflammation. That is, by forming covalent complexes with differing proteins,  $\alpha_2$ M may be acting as a carrier or adaptor molecule that mediates rapid

- 15 internalization of these proteins. The receptor recognition site at the C-terminus of each  $\alpha_2$ M subunit is masked until after proteolytic activation (13), during which complex formation with a variety of proteins can occur. Because complex formation depends upon proteolytic activity, which is usually tightly controlled *in vivo*, the proteins carried by  $\alpha_2$ M into the macrophages would be limited to those  
20 that are present in areas of enhanced proteolytic activity, as might be expected in areas of inflammation. Since  $\alpha_2$ M can be activated by completely unrelated proteinases, both pathogen- and host-derived proteinases could serve this purpose. This proteolytic activity may also serve to cleave large Ag into smaller fragments that can enter the trap, while the covalent binding mechanism ensures capture of  
25 even the smaller peptides.

Human  $\alpha_2$ M is actively secreted by fibroblasts (62) and macrophages (63), which also secrete increased amounts of proteinases under inflammatory conditions (64).

- 30 The native form is present in plasma at levels greater than 3  $\mu$ M (18, 48), as well as in extravascular fluids (48). Changes in vascular permeability may result in leakage of serum proteins into sites of inflammation. Bovine  $\alpha_2$ M is present in

serum typically used to supplement culture media. Thus, native  $\alpha_2M$  would likely be present in areas of inflammation, as well as in many *in vitro* presentation systems. There are no known examples of complete  $\alpha_2M$  deficiency in mammals (57), lending further support to the importance of this conserved family of 5 thiolester-containing proteins.

In conclusion,  $\alpha_2M$  represents a naturally occurring protein capable of complexing with diverse proteins for rapid delivery into cells. It is abundant in extracellular fluids, where a wide range of proteinases can activate it to complex with potential 10 Ag. Its high affinity receptor will recognize only the "sprung trap" conformation ( $f-\alpha_2M$ ), allowing internalization of proteins prevalent in areas of increased proteolysis or inflammation. This study demonstrates that complexing Ag with  $\alpha_2M$ , using its intrinsic binding capacity, does indeed enhance both the rate and efficiency of Ag uptake and presentation by macrophages. On the basis of these 15 considerations and the above data, it is proposed that one possible function of the  $\alpha_2M$  receptor system may be enhancement of Ag capture, and therefore presentation, by reticuloendothelial APCs.

#### EXAMPLE 2

20

Example 1, above, demonstrates the effects of complexing  $\alpha_2M$  with hen egg lysozyme (HEL) upon Ag uptake and presentation to HEL-specific murine T-cell hybridomas (see also 20). The results indicated that  $\alpha_2M$  was capable of mediating receptor-facilitated Ag delivery to macrophages *in vitro*, decreasing both 25 the minimal Ag concentration needed for presentation and the time required for Ag internalization by about two orders of magnitude. In order to confirm the *in vivo* immune-enhancing activity of  $\alpha_2M$ , the abilities of human  $\alpha_2M$  ( $H\alpha_2M$ ) and the homologous rabbit  $\alpha_1M$  ( $R\alpha_1M$ ) to stimulate specific *in vivo* rabbit antibody responses to two complexed Ag -- HEL and porcine pancreatic elastase (PPE) -- 30 was tested. The results indicate that both  $H\alpha_2M$  and  $R\alpha_1M$  are capable of binding Ag and enhancing the adaptive immune response *in vivo*.

MATERIALS AND METHODS

*Materials.* HEL was purchased from Boehringer Mannheim (Indianapolis, IN). H $\alpha_2$ M was purified as previously described, employing extensive dialysis against dH<sub>2</sub>O to precipitate out the receptor-recognized f- $\alpha_2$ M conformational forms

- 5 [Example 1, (22,23,68)]. The purified H $\alpha_2$ M was >98% in the native s- $\alpha_2$ M conformation, as determined by 5,5'-dithiobis-(2-nitrobenzoic acid) titration of thiols released following proteolysis (20). R $\alpha_1$ M was purified by similar methods from citrated rabbit plasma (Pel-Freeze, Rogers, AR). R $\alpha_1$ M was not stable to freeze-thaw cycles, and was stored at 0°C. PPE of the highest available purity  
10 grade, low-endotoxin BSA, affinity-purified alkaline phosphatase-conjugated goat anti-[rabbit IgG], and p-nitrophenyl phosphate tablets were purchased from Sigma (St. Louis, MO). Endotoxin standards and Pyrotell *Limulus* amebocyte lysate reagents were obtained from Associates of Cape Cod (Woods Hole, MA).
- 15 *Ag Preparations.* The  $\alpha_2$ M-HEL-PPE complexes were prepared as described in Example 1 for  $\alpha_2$ M in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HEPES buffer) (see 68).. Incorporation ratios of 0.8 moles HEL/mole H $\alpha_2$ M and 0.14-0.32 moles HEL/mole R $\alpha_1$ M were achieved. H $\alpha_2$ M-methylamine was formed as previously described (31). both methylamine treatment and PPE treatment resulted in the  
20 complete transformation of s- $\alpha_2$ M to f- $\alpha_2$ M. All sterile Ag preparations contained less than 100 pg endotoxin/ml as determined by *Limulus* amebocyte lysate clotting times.

25 *Injection of Ag into NZW rabbits.* Pathogen-free NZW rabbits were purchased from Robinson's Services (Winston-Salem, NC) and housed in specific pathogen-free facilities at the Duke Vivarium. To investigate how complex formation with  $\alpha_2$ M affected the primary antibody response to HEL, twenty-two rabbits were injected with either HEL alone, H $\alpha_2$ M-HEL-PPE complexes, HEL mixed with  $\alpha_2$ M-methylamine (f- $\alpha_2$ M), HEL mixed with s- $\alpha_2$ M, or R $\alpha_1$ M-HEL-PPE  
30 complexes. In addition, six rabbits were injected with HEL emulsified in CFA. PPE that was previously inhibited with 3,4-dichloroisocoumarin (100  $\mu$ M) was

injected alone or mixed with BSA into four more rabbits to deliver the same amount of PPE as received by rabbits injected with  $\text{H}\alpha_2\text{M}$ -HEL-PPE complexes. All rabbits were bled before injection at Week 0 to obtain preimmune sera, which were used to verify the absence of preexisting antibodies directed against HEL,

5  $\text{H}\alpha_2\text{M}$ ,  $\text{R}\alpha_2\text{M}$ , or PPE. Blood was allowed to clot at 37°C, then spun down, and the sera were stored at -20°C until shortly before use.

*Antibody capture ELISAs.* Costar 96-well RIA/EIA plates were incubated overnight at 4°C with 8  $\mu\text{g}/\text{ml}$  of HEL, PPE, BSA,  $\text{R}\alpha_1\text{M}$ , or  $\text{H}\alpha_2\text{M}$ -methylamine,

10 in PBS pH 7.3. Coated plates were washed and blocked with PBS containing 5% Carnation non-fat dry milk and 0.05% Tween 80 (blocking buffer) for 2 h at room temperature. Plates were then incubated with 100  $\mu\text{l}$  of sera diluted in blocking buffer for 1 h at room temperature, followed by 100  $\mu\text{l}$  (1:2000 dilution) of alkaline phosphatase-coupled anti-[rabbit IgG]. After washing with blocking

15 buffer and then PBS, the substrate p-nitrophenyl phosphate (1 mg/ml in 0.1 M glycine, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{ZnCl}_2$ , pH 10.4) was added. Alkaline phosphatase activity was followed kinetically at 37°C using a THERMOmax™ microplate reader (Molecular Devices, Menlo Park, CA).

20 **RESULTS**

*Complexing HEL to  $\text{H}\alpha_2\text{M}$  enhances the primary antibody response.* After verifying that the preimmune serum from each of the rabbits lacked detectable antibodies against HEL, PPE, or  $\text{H}\alpha_2\text{M}$  (Table I, legend), the relative levels of anti-HEL IgG elicited in rabbits two weeks after the primary injection were

25 quantified by ELISA. All four rabbits that received  $\text{H}\alpha_2\text{M}$ -HEL-PPE complexes demonstrated much higher levels of HEL-specific antibody than did the rabbits that received equimolar amounts of free HEL (Figure 8). In fact, the IgG levels elicited by these complexes, which were injected in buffered saline, were comparable to those elicited by HEL emulsified in CFA (Figure 8).

TABLE I  
IgG Titers at Week 3\*

	Material Injected	Anti-HEL	Anti-PPE	Anti- $\alpha_2$ M	Anti-BSA
5	HEL in CFA	33000	0	0	--
	HEL in CFA	40000	--	--	--
	HEL in CFA	30000	--	--	--
	$\text{H}\alpha_2\text{M}$ -HEL-PPE	43000	23000	120000	--
	$\text{H}\alpha_2\text{M}$ -HEL-PPE	30000	10000	60000	--
	$\text{H}\alpha_2\text{M}$ -HEL-PPE	30000	--	--	--
10	$\text{H}\alpha_2\text{M}$ -HEL-PPE	32000	--	--	--
	HEL	4000	0	0	--
	HEL	5000	0	0	--
	HEL	3200	0	0	--
	HEL + s- $\alpha_2$ M	3000	--	110000	--
	HEL + s- $\alpha_2$ M	0	--	160000	--
15	HEL + f- $\alpha_2$ M	13000	0	90000	--
	HEL + f- $\alpha_2$ M	12000	0	140000	--
	PPE	--	700	--	--
	PPE	--	3000	--	--
	PPE + BSA	--	3000	--	10000
	PPE + BSA	--	700	--	10000
20					

\* Rabbits were injected with the equivalent of 125  $\mu\text{g}$  HEL complexed to  $\text{H}\alpha_2\text{M}$  at Weeks 0 and 2. S- $\alpha_2\text{M}$  and f- $\alpha_2\text{M}$  (methylamine-treated) refer to specific

25 conformational forms of  $\text{H}\alpha_2\text{M}$ ; all the complexes consisted of HEL bound to f- $\alpha_2\text{M}$  (proteinase-activated). Titers reflect the maximum dilution factor that yielded substrate hydrolysis rates of 1 mOD/min. Sera that failed to produce this

hydrolysis rate in a 31.6-fold dilution were assigned zero titers. Preimmune sera from every rabbit displayed zero titers against HEL, PPE, or  $\text{H}\alpha_2\text{M}$  (average hydrolysis rates of 0.132 mOD/min  $\pm$  SD of 0.09, 0.196  $\pm$  0.16, and 0.275  $\pm$  0.10 respectively).

Because the rabbits also demonstrated a vigorous response to the H $\alpha_2$ M part of the complex (Table I), the enhanced anti-HEL response could have been due to general stimulation of the immune system. Additionally, ligation of the  $\alpha_2$ M receptor could have affected macrophage function directly. To investigate these 5 possibilities, rabbits were injected with HEL mixed with either s- $\alpha_2$ M or f- $\alpha_2$ M. The primary IgG response to HEL mixed with s- $\alpha_2$ M was not significantly different from that elicited by HEL alone. However, mixing HEL with the receptor-recognized conformational form resulted in an intermediate level of response (Figure 8). Both s- $\alpha_2$ M and f- $\alpha_2$ M elicited vigorous anti-H $\alpha_2$ M 10 responses (Table I), verifying that the antigenicity of H $\alpha_2$ M did not by itself explain the intermediate response of HEL plus f- $\alpha_2$ M.

The reciprocal dilution of each serum sample that yielded substrate hydrolysis rates of 1 milli-OD units per min (1mOD/min) during ELISA was defined as the 15 end titer. At Ag doses equivalent to 125  $\mu$ g HEL, both emulsification in CFA and APC targeting via the  $\alpha_2$ M receptor resulted in about a 10-fold increase in titers compared to free HEL (Figure 8 and Table I). Use of a lower Ag dose (40-50  $\mu$ g HEL equiv.) resulted in an even more dramatic enhancement of the primary 20 response (Figure 9A). At this dose, only one out of four rabbits injected with HEL demonstrated a detectable titer. All three of the rabbits receiving H $\alpha_2$ M-HEL-PPE complexes responded, demonstrating 100-fold higher peak titers (Figure 9A). At this lower dose, mixing HEL with f- $\alpha_2$ M yielded only a slight increase in peak titer compared with HEL alone, and this increase was delayed in its onset. 25 *H $\alpha_2$ M-complexed HEL elicits higher IgG titers after multiple injections.* End titers for each of the protein components that was injected -- HEL, H $\alpha_2$ M, PPE, or BSA -- were calculated for each rabbit at Week 3, after injections with the different Ag preparations (125  $\mu$ g HEL equiv.) at Week 0 and 2 (Table I). Titers for the four 30 rabbits that received either free HEL or HEL mixed with s- $\alpha_2$ M were indistinguishable. The rabbits that received H $\alpha_2$ M-complexed HEL displayed consistently higher anti-HEL titers, as did the rabbits that received CFA (Table I).

Titers were also calculated for an arbitrarily defined endpoint, 5mOD/min, with equivalent results (not shown).

*R $\alpha_1$ M-HEL complexes also elicit efficient primary IgG responses.* The control

- 5 experiments described above establish that Ag complexed directly with  $\alpha_2$ M demonstrates the greatest enhancement in immunogenicity. However, it is possible that the Ag are carried into cells through another uptake mechanism after forming immune complexes with anti-H $\alpha_2$ M antibodies. Although there were no detectable anti-H $\alpha_2$ M titers in the preimmune sera (Table I, legend), a particularly rapid  
10 immune response directed against H $\alpha_2$ M may have resulted in some immune complex formation during the second injection at Week 2. Thus, we developed a purification procedure for a rabbit homologue of H $\alpha_2$ M (R $\alpha_1$ M) and studied the development of a primary IgG response following a single injection equivalent to 40  $\mu$ g of HEL.

15

As with H $\alpha_2$ M, complexes involving the R $\alpha_1$ M elicited titers equivalent to those achieved by emulsification in CFA (Figure 9B). After the peak response, titers elicited by R $\alpha_1$ M complexes fell more rapidly than those elicited by CFA, perhaps reflecting the depot property of the water in oil CFA emulsion. Nevertheless,

- 20 mean titers were still at least 200-500 times higher than those elicited by free HEL during Weeks 2 and 3 (Figure 9B).

After their titers returned to baseline values (Week 5), the rabbits that received free HEL or R $\alpha_1$ M-HEL-PPE were rechallenged with a 10  $\mu$ g dose of free HEL in

- 25 saline. None of the four rabbits that had been primed initially using free HEL responded. Rabbits that had been primed with R $\alpha_2$ M complexes all responded with titers of 2000 one week after the secondary challenge (not shown).

*Complexing a second Ag to H $\alpha_2$ M also leads to enhanced antibody formation.* The

- 30 specific IgG response to the proteinase, PPE, that was complexed to  $\alpha_2$ M was also examined. PPE was inhibited irreversibly by the active site-directed inhibitor 3,4-

dichloroisocoumarin before being injected into rabbits or coated onto ELISA plates. As with HEL, anti-PPE antibody levels were enhanced by coupling to H $\alpha_2$ M (Figure 10). The addition of BSA (adjusted to deliver the same mass of foreign protein as in the H $\alpha_2$ M complexes) had no effect on the antibody response 5 to PPE (Figure 10), even though it resulted in a good anti-BSA response (Table I).

### DISCUSSION

Although its unique capacity for inhibiting a wide range of unrelated proteinases has been a major focus of study, there are indications that the reaction of  $\alpha_2$ M

10 with proteinases may subserve broader roles. In addition to binding potential Ag and delivering them to macrophages,  $\alpha_2$ M has been reported to bind many growth regulating substances [Reviewed in 90-92]. Although growth factor binding may simply reflect its general ability to interact with many different proteins,  $\alpha_2$ M could potentially alter their biodistributions and activities. In addition, we have  
15 recently shown that the binding of f- $\alpha_2$ M to its receptors on macrophages results in several intracellular signalling events (93). These observations suggest that  $\alpha_2$ M may function as a sensor for situations involving increased proteolysis.

Thus, although the present invention is not intended to be limited by any particular  
20 theory, there are several potential *in vivo* mechanisms by which  $\alpha_2$ M could have influenced antibody production against the two bound proteins, HEL and PPE. Since the responses to Ag covalently complexed to  $\alpha_2$ M were much greater than those elicited by control mixtures of Ag with  $\alpha_2$ M, the major mechanism probably involved Ag targeting the APCs for enhanced uptake via the  $\alpha_2$ M receptor (Figure  
25 11), as was demonstrated *in vitro* [Example 1, (68)]. The high efficiency of f- $\alpha_2$ M clearance by this receptor, which contains internalization signals homologous to those of the LDL receptor (10, 11, 94), together with the lack of pre-existing anti- $\alpha_2$ M titers, make the formation of immune complexes unlikely. Moreover, homologous injection of R $\alpha_1$ M-HEL complexes into rabbits elicited IgG responses  
30 comparable to those elicited by injections of H $\alpha_2$ M-HEL complexes in every respect (Figure 9).

Interestingly, at the higher 125 µg equivalent doses, receptor-recognized f- $\alpha_2$ M also appeared to enhance IgG production to an intermediate degree, independently of covalent crosslinking (Figure 8; Table I). This intermediate effect was not seen *in vitro* [Example 1, (68)]. This was not due to the trivial explanation that the

- 5 overall antigenic load on the immune system stimulated the effectiveness of antibody production nonspecifically since neither the addition of s- $\alpha_2$ M nor 6 mg of BSA influenced IgG production against free Ag despite the significant immunogenicity of both antigens (Figs. 8 and 10; Table I).

- 10 Receptor ligation of f- $\alpha_2$ M initiates macrophage second messenger responses, including effects on inositol trisphosphates, intracellular Ca<sup>2+</sup>, cAMP, prostaglandins and protein kinase C (93, 95, Example 4). F- $\alpha_2$ M has been described as chemotactic for macrophages (96), eliciting a "spread out" macrophage morphology (52), and an intracellular signalling pattern reminiscent of chemoattractants (93, 97). Thus, the addition of higher doses of f- $\alpha_2$ M *in vivo* may have affected leukocyte mobility. Other effects of  $\alpha_2$ M-elicited second messengers may include APC activation and regulation of costimulatory signals and molecules.
- 15
- 20 Another potential mechanism might involve the binding of endogenous cytokines; however, this explanation is kinetically improbable. Although f- $\alpha_2$ M is cleared rapidly *in vivo* [ $t_{1/2} = 2-4$  min (9, 16)], many cytokines appear to require much longer periods of incubation to form noncovalent or disulfide-linked complexes [ $t_{1/2} = 1-2$  h (66, 16, 98)].

25

Based upon these data, as well as the distinct properties of  $\alpha_2$ M discussed above, a potential role for  $\alpha_2$ M in Ag processing is shown in Figure 11. Areas of inflammation contain high levels of s- $\alpha_2$ M both from increased plasma extravasation and from increased local synthesis (99). Among the cells that

- 30 secrete  $\alpha_2$ M are fibroblasts and macrophages (62, 63). These sites would also possess enriched levels of inflammatory proteinases and foreign proteins. S- $\alpha_2$ M,

which is not receptor-recognized, would react with the proteinases in the presence of these antigenic proteins, resulting in receptor-recognized f- $\alpha_2$ M forms carrying a mixture of proteins. Upon endocytosis into macrophages or other APCs that express the receptor, the bound proteins would be processed and presented for surveillance by T-cells. Among the cells that express the  $\alpha_2$ M receptor are monocytes/macrophages (41) fibroblasts (100, 101), and dermal dendritic cells (102). Given the common stem cell ancestry of monocytes and Langerhans dendritic cells (103), and the fibroblastic nature of the lymph node reticulum cell that gives rise to follicular dendritic cells (73), these nonphagocytic APCs may also express the  $\alpha_2$ M receptor.

In summary, complexes formed during proteolytic activation of  $\alpha_2$ M undergo receptor-mediated endocytosis into macrophages, dendritic cells, and other cells bearing the  $\alpha_2$ M receptor, resulting in enhanced cellular humoral immune responses *in vitro* and *in vivo*. In addition, ligation of the  $\alpha_2$ M receptor itself may also affect the immune response *in vivo*. Species specific or autologous  $\alpha_2$ M may prove to be an effective, nonirritating alternative to traditional adjuvants.

### EXAMPLE 3

20

The  $\alpha_2$ M trap has been considered to prevent diffusion of fairly large molecules, e.g., proteinases. This conclusion results from the observation that anything larger than about 10,000 to 15,000 molecular weight cannot access the active site of a trapped proteinase which is believed to be deeply buried within the  $\alpha_2$ M molecule. As discussed in the Background of the Invention, this steric inhibition that results from trapping is the mechanism by which  $\alpha_2$ M inhibits proteinases.

However, the "trap" may be more "leaky" than previously thought. Nonproteolytic proteins up to about 22,000 to 23,000 molecular weight can fall out of the closed trap if they are not covalently bound. The present Example demonstrates that  $\alpha_2$ M can protect bound antigens.

Insulin was labelled with  $^{125}\text{I}$ . The labelled insulin ( $1.4 \mu\text{m}$ ) was complexed with  $\alpha_2\text{M}$  ( $1.4 \mu\text{m}$ ) by treatment with elastase ( $2.5 \mu\text{m}$ ) for 10 min. Then the remaining elastase was irreversibly inhibited by treatment with dichloroisocoumarin (DCI), an active site directed inhibitor. These reaction

- 5   solutions were termed  $\alpha_2\text{M}$ -insulin complexes. After this treatment, all of the  $\alpha_2\text{M}$  is in the fast form, therefore additional elastase would not be inhibited by residual  $\alpha_2\text{M}$ . The following reaction solutions were prepared: (A)  $^{125}\text{I}$  insulin alone; (B) a preformed  $\alpha_2\text{M}$ -insulin complex, incubated for 10 min with elastase; (C) a preformed  $\alpha_2\text{M}$ -insulin complex treated for 2 min with elastase; (D)  $\alpha_2\text{M}$  and  
10   insulin treated with elastase for 10 min (*i.e.*, the complex formation reaction); (E) insulin treated with elastase for 2 min, followed by addition of  $\alpha_2\text{M}$  and incubation for an additional 10 min; (F) insulin treated with elastase for 10 min, followed by addition of  $\alpha_2\text{M}$  and incubation for an additional 10 min; (G) insulin treated with elastase for 10 min.

15

- The results of this experiment are shown in Figure 12, which is an autoradiogram of a non-reduced SDS-PAGE gel in which the lanes correspond to the reaction solutions described above.  $^{125}\text{I}$ -insulin-labelled  $\alpha_2\text{M}$  is found in lanes B, C, D, E and F. Lane A contains non-degraded insulin, and lane G contains both non-degraded and degraded insulin. The insulin complexed with  $\alpha_2\text{M}$  was slightly degraded with 10 min exposure to elastase (lane B), but not with 2 min exposure (lane C). In contrast, insulin reacted with elastase prior to complex formation was significantly degraded (lanes E and F). A small amount of degradation was found when insulin was reacted with elastase and  $\alpha_2\text{M}$  at the same time (lane D).

25

These results demonstrate that the peptide insulin was significantly protected from proteolysis when found in a complex with  $\alpha_2\text{M}$ .

EXAMPLE 4

This Example demonstrates the ability of the RBF of  $\alpha_2$ M to activate the  $\alpha_2$ M signalling receptor. Evidence for signalling receptor activation included increase  
5 in intracellular calcium concentration; cholera toxin-induced ADP-ribosylation of the 43 kDa G-protein; and protein kinase C (PKC) activation, as measured by histone phosphorylation and movement of tritium labelled phorbol dibutyrate [ $^3$ H]-PDBu, a DAG analog, to the cell membrane.

10 MATERIALS AND METHODS

Cell culture, preparation of  $\alpha_2$ M, measurement of intracellular calcium levels, and quantification of inositol phosphates were performed as described in Misra et al., 1993, *BIOCHEM. J.*, 290:885-891.

- 15 *PKC Measurements.* Thioglycollate-elicited murine peritoneal macrophages were plated at a density of  $6 \times 10^6$  cells in 35 mm Petri dishes containing RPMI 1640 medium. After 16 to 18 h, the medium was aspirated and the cells washed three times in HHBSS. Native  $\alpha_1$ I<sub>3</sub>,  $\alpha_1$ I<sub>3</sub>-methylamine or buffer each containing 75  $\mu$ M Ca<sup>2+</sup> was then added. The cells were incubated for 20 min at 37°C in a  
20 humidified incubator under 5% CO<sub>2</sub>. The reaction was terminated by aspirating the buffer and addition of a volume of buffer containing 20 mM HEPES, 10 mM EGTA, 2 mM EDTA, 5 mM DTT, 20  $\mu$ g/ml leupeptin, 1 mM PMSF, 0.25M sucrose, 1% nonidet P40, pH 7.4. The cells were scraped, transferred to tubes and sonicated on ice (five 10 s bursts with 30 s intervals). The sonicate was left  
25 on ice for 20 min and then centrifuged at 100,000 x g for 60 min at 4°C. The supernatant was then applied to a DE 52 column pre-equilibrated with 20 mM HEPES, 10 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, 20  $\mu$ g/ml leupeptin, pH 7.4. The column was eluted with the same buffer containing 300 mM NaCl at a flow rate of 8 ml/h. The PKC activity was determined by histone  
30 III's phosphorylation using [ $^{32}$ P]- $\gamma$ -ATP (Sahyoun, N.E. et al., 1989, *J. BIOL.*

*CHEM.* **264:**1062-1067) and by [<sup>3</sup>H]PDBu binding (Misra and Sahyoun, 1987, *BIOCHEM. BIOPHYS. RES. COMM.* **145:**760-767).

- 5 The 20 kDa RBF was obtained as described in Salvesen et al. ((1992), *FEBS* **313:**198-202). This is the C-terminal RBF of rat  $\alpha_1$ M expressed in *E. coli*. The purified protein was passed serially through three 2 ml Detoxi-Gel columns (Pierce Immunochemicals) with regeneration of the columns between runs, until endotoxin levels were < 10 pg/ml in the 1.3  $\mu$ m stock solution as determined by Pyrotell
- 10 *Limulus* amebocyte clotting times (Association of Cape Cod, Woods Hole, MA) using a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA) (see Example 1, *supra*).

#### RESULTS AND DISCUSSION

- 15 The results shown in Figure 13 demonstrate that the 20 kDa RBF expressed in *E. coli* can stimulate an increase in intracellular calcium levels, as is found with f- $\alpha_2$ M ( $\alpha_2$ M treated with methylamine to activate it). Interestingly, the increase in intracellular calcium concentration continued for a significant period after treatment with the RBF, but not after treatment with f- $\alpha_2$ M. This indicates that
- 20 RBF mediates signal transduction for a longer period, perhaps because it is not removed from the cell surface as rapidly via endocytosis. Additional experiments confirmed this observation.

- Although pre-treatment with either  $\alpha_2$ M-methylamine or RBF eliminates a
- 25 subsequent response to the other factor administered within about 5 minutes (data not shown), the 39 kDa receptor-associated protein (RAP39) that blocks the binding of  $\alpha_2$ M to the endocytic receptor had no effect upon column signals. Tetrameric  $\alpha_2$ M-methylamine and monomeric RBF were found to have roughly equivalent pharmacological potencies on a molar basis with regard to  $\text{Ca}^{2+}$
  - 30 mobilization (Figure 14).

Similar results were observed when evaluating histone phosphorylation as a marker for cellular protein kinase-C (PKC) activation. The data in Figure 15A show that the RBF stimulates increased histone phosphorylation compared to buffer. The PKC inhibitor staurosporin inhibits this increase. Increased histone

- 5 phosphorylation, that could be inhibited with staurosporin, was observed upon activation with  $\alpha_2$ M-methylamine, rat  $\alpha_1$ -inhibitor<sub>3</sub> (a monomeric  $\alpha_2$ M homolog), and RBF. Notably, RBF present at a 5-fold lower concentration than either the tetrameric or monomeric proteins (40 nM for RBF versus 200 nM for the proteins) was nevertheless comparably effective at enhancing PKC activation.

10

- Movement of the diacylglycerol (DAG) [<sup>3</sup>H]-PDBu to cell membranes also demonstrates PKC activation. Increased binding of the labelled DAG analog in TG-elicited murine macrophages was evaluated after treatment with buffer, slow and fast  $\alpha_2$ M, slow and fast  $\alpha_1$ -inhibitor<sub>3</sub>, and the 20 kDa RBF C-terminal  
15 fragment from rat  $\alpha_1$ M. The slow form macroglobulins do not bind receptor, so these samples serve as negative controls. The fast form of both intact ligands was formed by treatment with methylamine.

- The results of this experiment are shown in Figure 16. The intact fast form  
20 ligands  $\alpha_2$ M (a homotetramer) and  $\alpha_1$ -inhibitor<sub>3</sub> (a monomer) both stimulated movement of [<sup>3</sup>H]-PDBu to cell membranes when compared to buffer and the slow form (non-receptor binding) controls. Likewise, increased movement of the DAG analog to membranes resulted from treatment with the RBF. Most remarkably, comparable PKC activation was effected by a much lower concentration of RBF  
25 than the intact ligands, as was observed in the histone phosphorylation assay. Specifically, the amount of label moved to the membrane was about the same with 40 nM of the RBF and 200 nM of either intact ligand.

- The RBF also stimulated increased cholera toxin-induced ADP-ribosylation of the  
30 43 kDa G-protein found in macrophages when compared to buffer (data not shown).

These results indicate that the 20 kDa receptor binding fragment binds to a signalling receptor for  $\alpha_2$ M on macrophages that is novel and distinct from the previously described endocytic  $\alpha_2$ M receptor, although the two receptor proteins may interact. Binding to this second receptor stimulates cellular activation. From

- 5 the range of assays performed, it appears that all of the signal events resulting from  $\alpha_2$ M binding to APCs occur upon binding the 20 kDa fragment as well. Moreover, the 20 kDa fragment was found to be as potent on a molar basis as intact f- $\alpha_2$ M at stimulating increased concentration of  $[Ca^{2+}]_i$ , and about 5-fold more potent on a molar basis at PKC activation than either f- $\alpha_2$ M and f- $\alpha_1$ -inhibitor<sub>3</sub>.

- 10  
15 The following is a listing of the publications referred to in the foregoing specification, with numbers corresponding to those presented hereinabove. Each of the following references, as well as the references cited throughout this specification, is hereby incorporated herein in its entirety.

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5 Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early biopotent CD34+ progenitors in human bone marrow. *J. Immunol.* 149:2681.
- 10 This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and  
15 all changes which come within the meaning and range of equivalency are intended to be embraced therein.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Duke University (except for the U.S.)

Pizzo, Salvatore V.  
Chu, Charleen T.  
Oury, Timothy D.

(ii) TITLE OF INVENTION: IMMUNE RESPONSE MODULATOR COMPLEX, AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: David A. Jackson, Esq.
- (B) STREET: 411 Hackensack Avenue
- (C) CITY: Hackensack
- (D) STATE: New Jersey
- (E) COUNTRY: USA
- (F) ZIP: 07601

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: WO to be assigned
- (B) FILING DATE: 20-DEC-1993
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/992,899
- (B) FILING DATE: 18-DEC-1992

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Jackson Esq., David A.
- (B) REGISTRATION NUMBER: 26,742
- (C) REFERENCE/DOCKET NUMBER: 931-1-008 PCT/CIP

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- (A) TELEPHONE: (201) 487-5800
- (B) TELEFAX: (201) 343-1684
- (C) TELEX: 133521

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Gly Gly Gly Cys Gly Gly Glu Gly Gly Gly Gly Tyr Gly Gly Gly  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Hart, Mary K.

Weinhold, Kent J.

Scearce, Richard M.

Washburn, Eileen M.

Clark, Cynthia A.

Palker, Thomas J.

Haynes, Barton F.

(B) TITLE: Priming of anti-human immunodeficiency virus  
(HIV) CD8+ cytotoxic T cells in vivo by  
carrier-free HIV synthetic peptides

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 88

(F) PAGES: 9448-9452

(G) DATE: November-1991

(K) RELEVANT RESIDUES IN SEQ ID NO:2: FROM 1 TO 40

82

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Cys Thr Arg Pro Asn Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro  
20                   25                   30

Gly Arg Ala Phe Tyr Thr Thr Lys  
35                   40

WHAT IS CLAIMED IS:

- 1 1. A method of inducing a modified immune response to an antigen comprising administering an antigen and  $\alpha_2$ -macroglobulin ( $\alpha_2$ M) or a fragment thereof, which  $\alpha_2$ M or fragment thereof is capable of binding a receptor for  $\alpha_2$ M, in an amount sufficient to induce a modified immune response to said antigen.
- 1 2. A method in accordance with Claim 1 wherein the antigen is in a complex with  $\alpha_2$ M or the fragment thereof.
- 1 3. A method in accordance with Claim 2 wherein the antigen is covalently bound to the  $\alpha_2$ M or fragment thereof.
- 1 4. A method in accordance with Claim 1 wherein the antigenicity of an epitope region on the antigen increases.
- 1 5. A method in accordance with Claim 1 wherein the antigenicity of an epitope region on the antigen decreases.
- 1 6. A method in accordance with Claim 1 wherein the complex is polyvalent and the reaction increases the antigenicity of a subordinate epitope while downregulating the antigenicity of an immunodominant epitope on the antigen.
- 1 7. A method in accordance with Claim 1 wherein the modified immune response is an enhanced antibody response.
- 1 8. A method in accordance with Claim 1 wherein the antigen is selected from the group consisting of peptides, proteins, carbohydrates, cytokines, growth factors, hormones, enzymes, toxins, anti-sense RNA, other drugs and oligonucleotides.

1    9.    A method in accordance with Claim 1 wherein the  $\alpha_2M$  fragment comprises  
2    a carboxyl terminal portion of said  $\alpha_2M$  containing the receptor binding region.

1    10.   A method in accordance with Claim 9 wherein the fragment contains a cis-  
2    dichlorodiamine platinum reactive/oxidation sensitive residue.

1    11.   A method in accordance with Claim 10 wherein the fragment has a  
2    molecular weight of approximately 40 kilo-Daltons.

1    12.   A method in accordance with Claim 9 wherein the fragment lacks a cis-  
2    dichlorodiamine platinum reactive/oxidation sensitive residue.

1    13.   A method in accordance with Claim 12 wherein the fragment has a  
2    molecular weight of approximately 20-30 kilo-Daltons.

1    14.   A method in accordance with Claim 9 wherein said fragment comprises a  
2    plurality of said carboxyl terminal portions.

1    15.   A method in accordance with Claim 9 in which the antigen and the  
2    carboxyl terminal portion of  $\alpha_2M$  are a fusion protein.

1    16.   An antibody which recognizes an epitope on an antigenic molecule, said  
2    antigenic molecule having been reacted with a complex between an antigen and  
3     $\alpha_2M$  or an active fragment thereof, in an amount effective for modifying the  
4    antigenicity of the antigen, said antibody not being produced in the absence of  
5    such reaction.

1    17.   An antibody in accordance with Claim 16 which is polyclonal, monoclonal  
2    or chimeric.

- 1 18. An immunogen comprised of an antigenic molecule having at least one
- 2 epitope and in a complex with  $\alpha_2M$  or a fragment, which  $\alpha_2M$  or fragment thereof
- 3 is capable of binding a receptor for  $\alpha_2M$ .
- 4
- 1 19. A method of rendering an epitope on an antigen recognizable by the
- 2 immune system, which epitope does not substantially induce an immune response
- 3 under normal conditions, comprising:
  - 4 reacting the antigen molecule with  $\alpha_2M$  or an active fragment thereof to
  - 5 form a complex;
  - 6 exposing an antigen presenting cell having MHC to the complex; and
  - 7 contacting said antigen presenting cell with lymphocytes.
- 1 20. An antigen presentation complex comprised of:
  - 2 (a) an antigen presenting cell having major histocompatibility complex on
  - 3 the cell surface, and
  - 4 (b) an antigen which is comprised of an epitope presented in the context of
  - 5 MHC on the antigen presenting cell, which antigen has been reacted to form a
  - 6 complex with  $\alpha_2M$  or a fragment thereof, which  $\alpha_2M$  or fragment thereof is
  - 7 capable of binding a receptor for  $\alpha_2M$ .
- 1 21. A vaccine comprising an antigen and  $\alpha_2M$  or a fragment thereof, which
- 2  $\alpha_2M$  or fragment thereof is capable of binding a receptor for  $\alpha_2M$ .
- 1 22. The vaccine of Claim 21 wherein the antigen is in a complex with  $\alpha_2M$  or
- 2 the fragment thereof.
- 1 23. The vaccine of Claim 22 wherein the antigen is covalently bound to the
- 2  $\alpha_2M$  or fragment thereof.

1 24. The vaccine of Claim 21 wherein said fragment comprises the carboxyl  
2 terminal portion of said  $\alpha_2M$  containing the receptor binding region.

1 25. A vaccine of Claim 24 wherein the fragment contains a cis-dichlorodiamine  
2 platinum reactive/oxidation sensitive residue.

1 26. A vaccine of Claim 25 wherein the fragment has a molecular weight of  
2 approximately 40 kilo-Daltons.

1 27. A vaccine of Claim 24 wherein the fragment lacks a cis-dichlorodiamine  
2 platinum reactive/oxidation sensitive residue.

1 28. The vaccine of Claim 27 wherein the fragment has a molecular weight of  
2 approximately 20-30 kilo-Daltons.

1 29. A method of determining T-lymphocyte levels, function or activity in a  
2 sample taken from a mammal comprising:

3 (a) exposing antigen presenting cells to an antigen, said antigen prepared  
4 as complex between the antigen and  $\alpha_2M$  or a fragment thereof, which  $\alpha_2M$  or  
5 fragment thereof is capable of binding a receptor for  $\alpha_2M$ ;

6 (b) combining said antigen exposed antigen presenting cells with T-  
7 lymphocytes taken from said mammal; and

8 (c) comparing the level, function or activity of said T-lymphocytes to a  
9 standard.

1 30. A method of producing T-lymphocytes which recognize an antigen,  
2 comprising:

3 administering to a mammal a T-lymphocyte priming effective amount of an  
4 antigen and  $\alpha_2M$  or a fragment thereof, which  $\alpha_2M$  or fragment thereof is capable  
5 of binding a receptor for  $\alpha_2M$ , and

6 harvesting said T-lymphocytes from said mammal.

- 1 31. A pharmaceutical composition comprised of antibodies as described in
- 2 Claim 16 in combination with a pharmaceutically acceptable carrier.
  
- 1 32. A biological composition comprised of T-lymphocytes which are produced
- 2 in accordance with Claim 30.
  
- 1 33. A method of treating or preventing an infectious disease, an autoimmune
- 2 disease or cancer in a mammalian patient in need of such treatment or prevention,
- 3 comprising administering to said patient an effective amount of an immunogen
- 4 comprised of an antigen, and  $\alpha_2M$  or a fragment thereof, which  $\alpha_2M$  or fragment
- 5 thereof is capable of binding a receptor for  $\alpha_2M$ , in an amount effective for
- 6 modifying the immune response to said antigen,
- 7       said immunogen being administered in an amount effective for treating or
- 8 preventing said infectious disease, autoimmune disease or cancer.
  
- 1 34. A method of treating or preventing an infectious disease, an autoimmune
- 2 disease or cancer in a mammalian patient in need of such treatment or prevention,
- 3 comprising administering to said patient an effective amount of antibodies of
- 4 Claim 15 specific for a disease-associated antigen,
- 5       said antibodies being administered in an amount effective for treating or
- 6 preventing said infectious disease, autoimmune disease or cancer.
  
- 1 35. A method of treating or preventing an infectious disease, an autoimmune
- 2 disease or cancer in a mammalian patient in need of such treatment or prevention,
- 3 comprising administering to said patient an effective amount of T-lymphocytes
- 4 produced according to the method of Claim 30, which T-lymphocytes are specific
- 5 for a disease-associated antigen,
- 6       said T-lymphocytes being administered in an amount effective for treating
- 7 or preventing said infectious disease, autoimmune disease or cancer.

1 36. A method for differentially modulating the activities of an endocytic  
2 receptor for  $\alpha_2M$  and a signalling receptor for  $\alpha_2M$  comprising contacting said  
3 receptors with a modified  $\alpha_2M$  or a fragment thereof, which  $\alpha_2M$  or fragment  
4 binds to at least one such receptor with a different affinity than a native  $\alpha_2M$  in a  
5 receptor-binding conformation.

1 37. The method of Claim 36 in which the  $\alpha_2M$  or fragment has been reacted  
2 with a reagent selected from the group consisting of an oxidant and cis-  
3 dichlorodiamine platinum.

1 38. The method of Claim 36 in which the fragment is a C-terminal fragment of  
2  $\alpha_2M$  lacking a cis-dichlorodiamine platinum reactive/oxidation sensitive residue.

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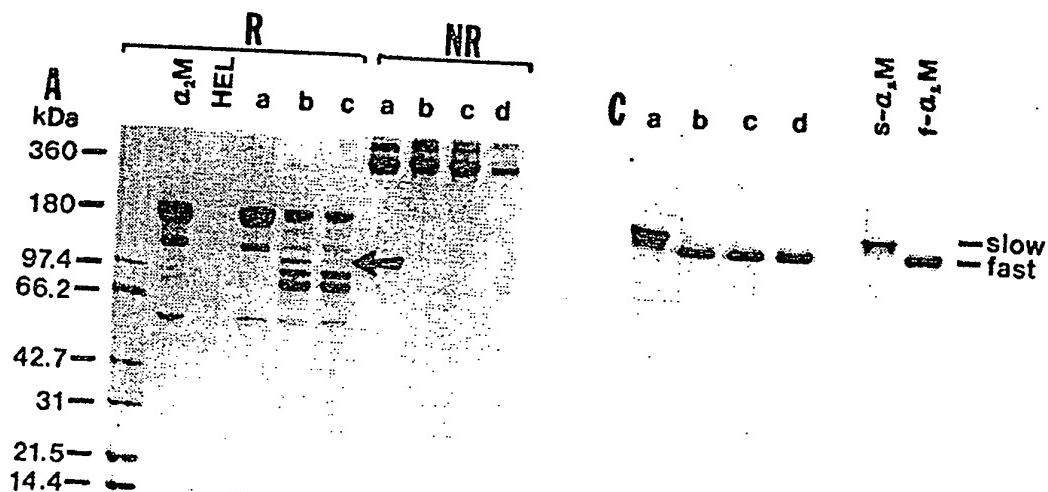


FIG. IA

FIG. 1C

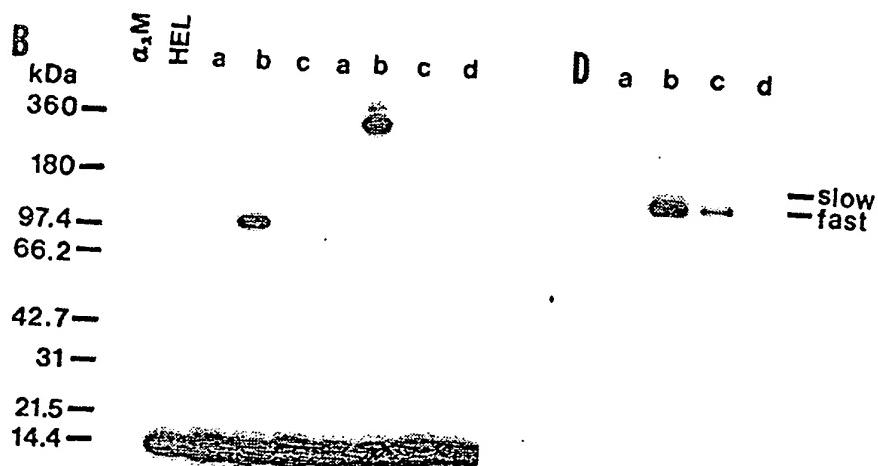


FIG.1B

FIG.1D

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FIG. 2A

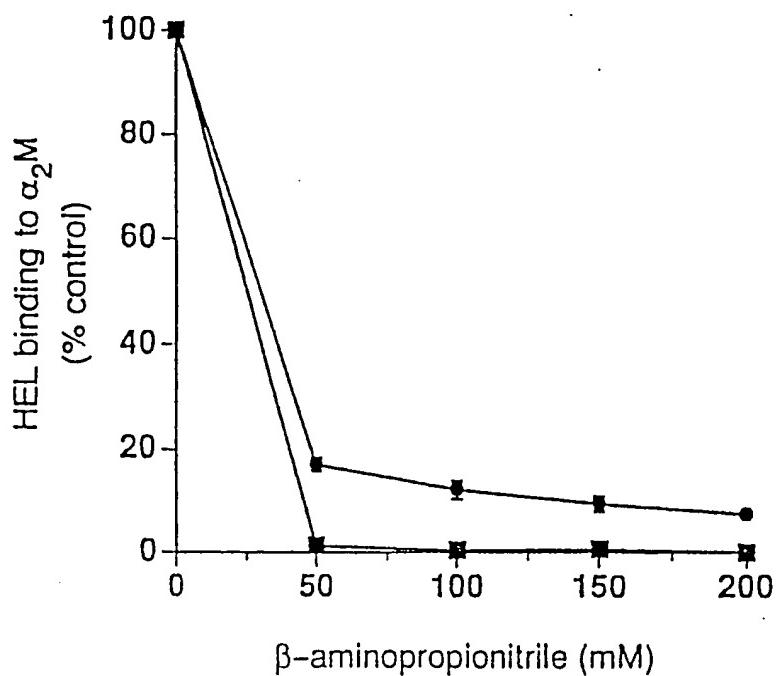
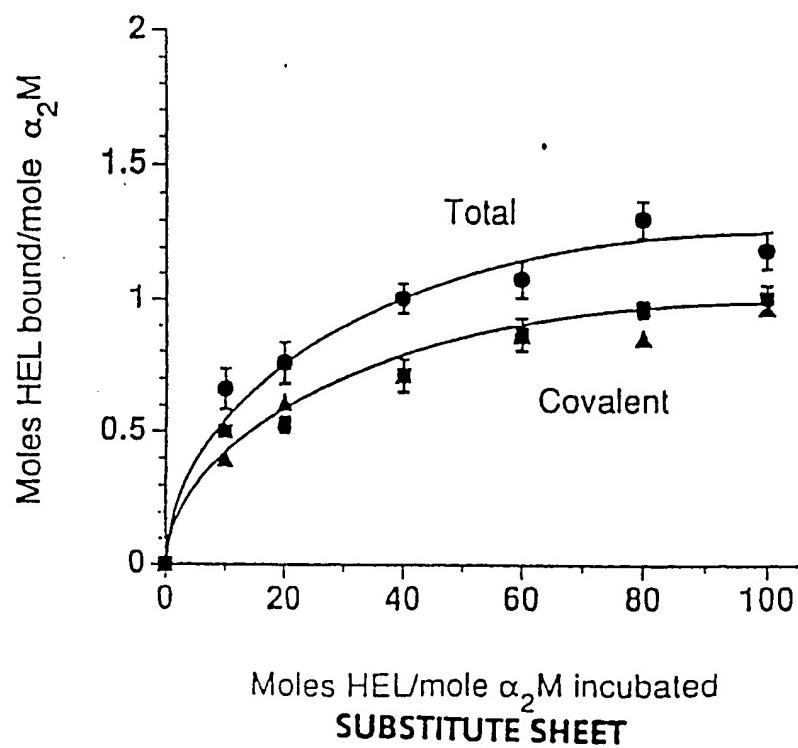
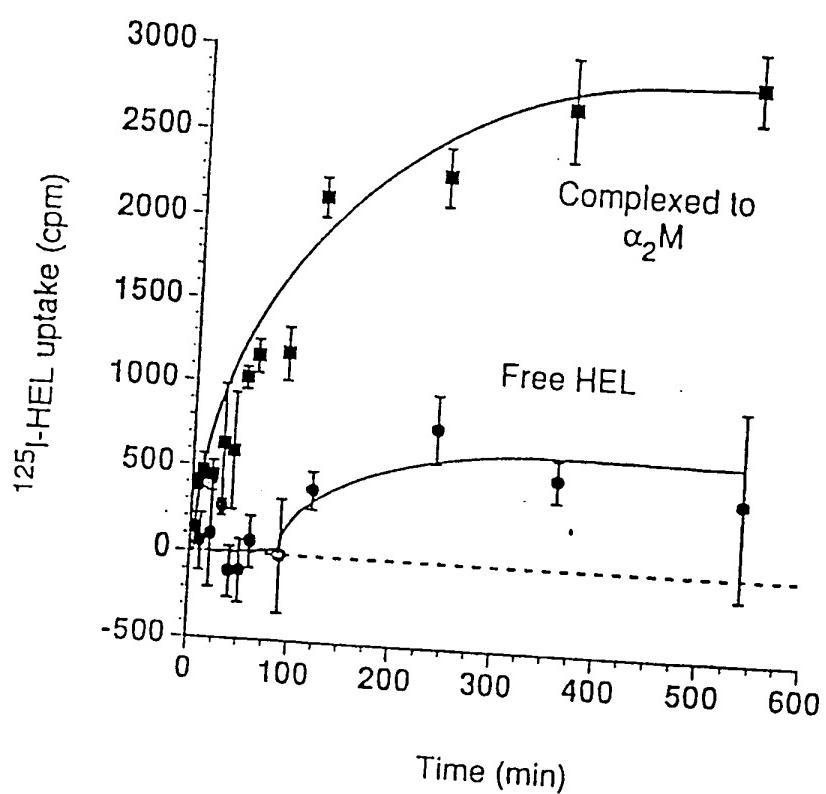


FIG. 2B



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FIG. 3



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FIG. 4B

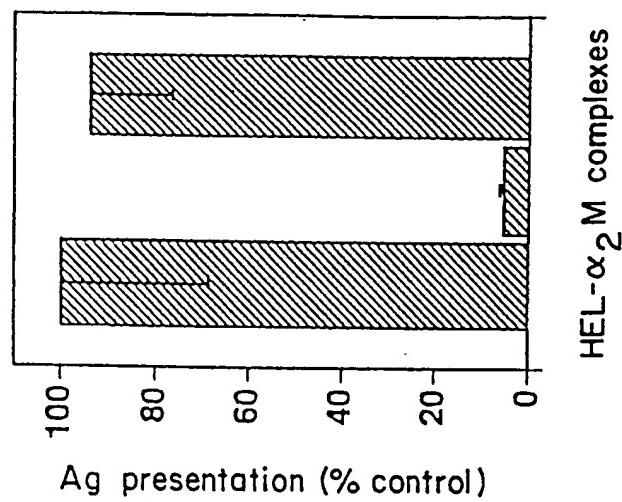
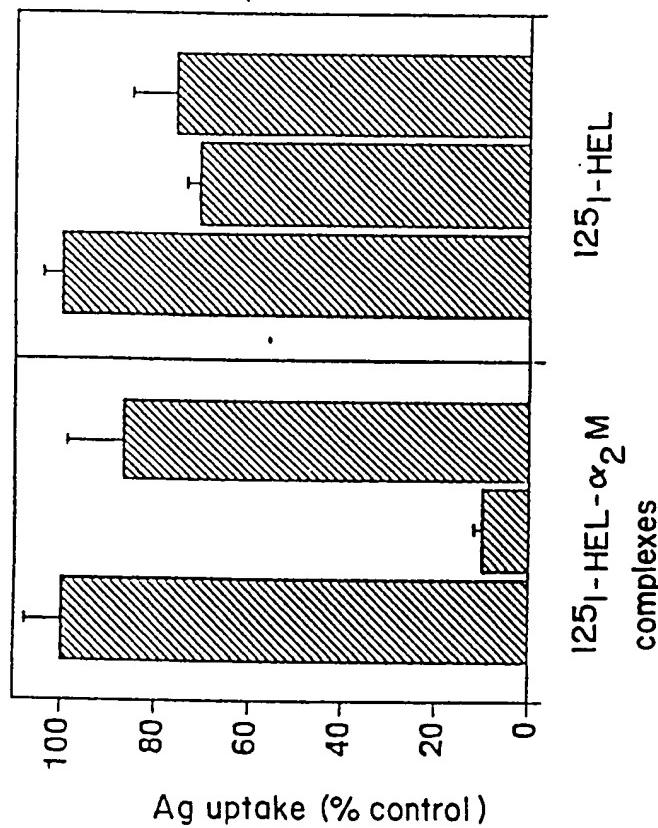
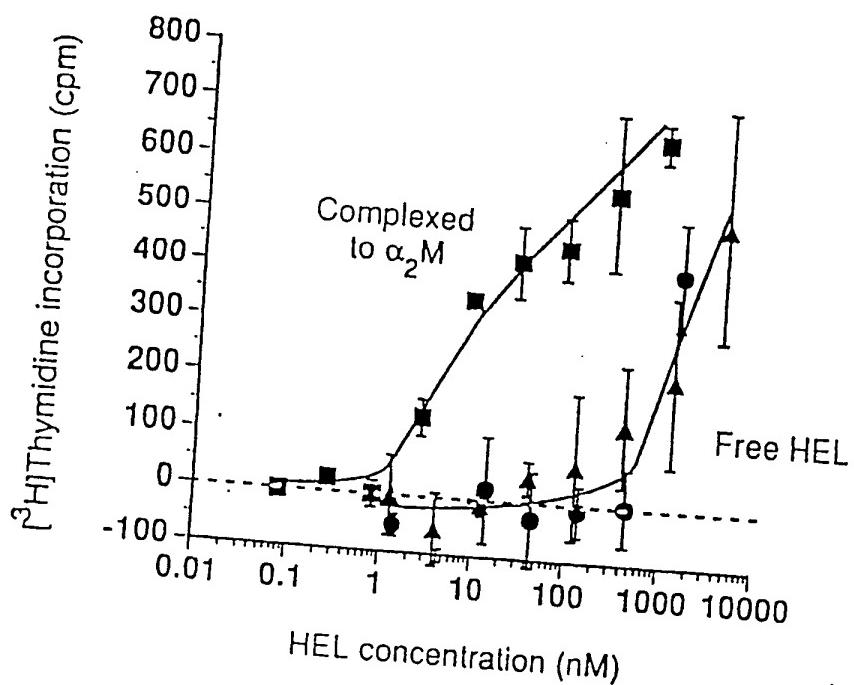


FIG. 4A



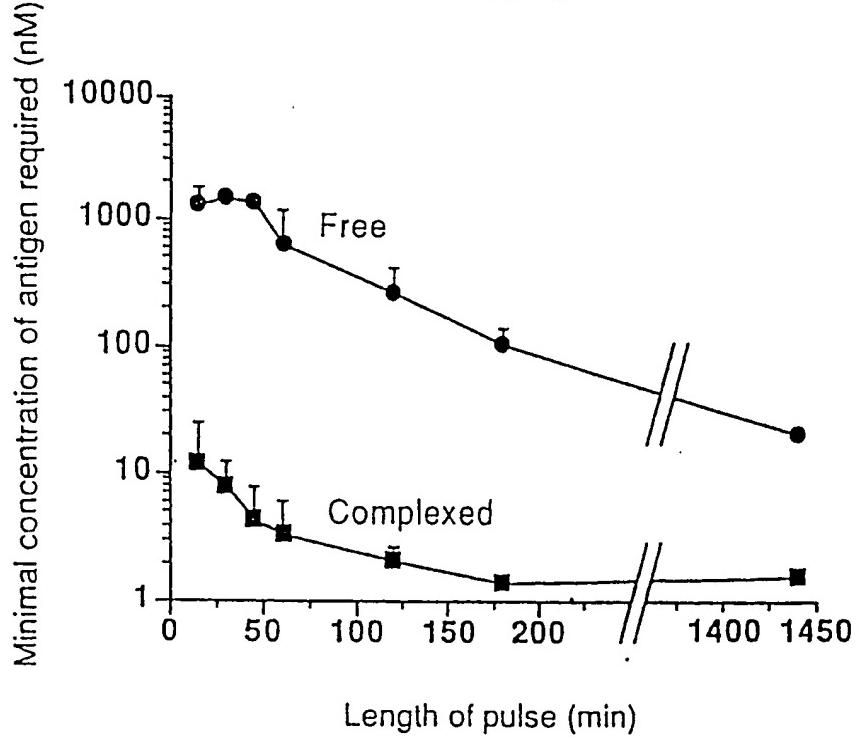
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FIG. 5



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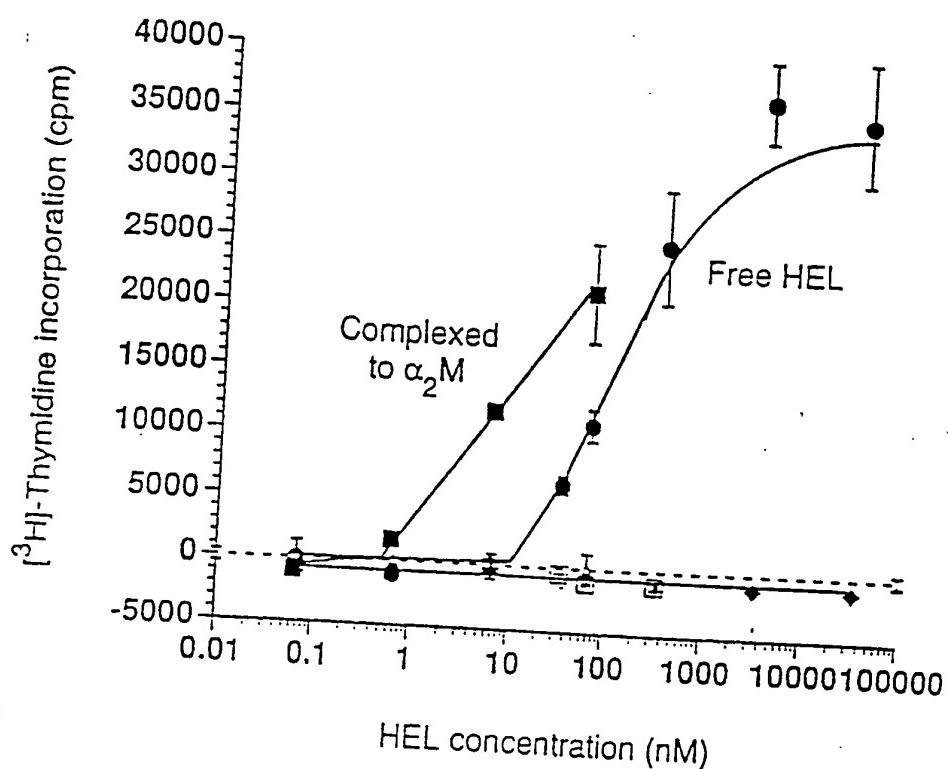
FIG. 6



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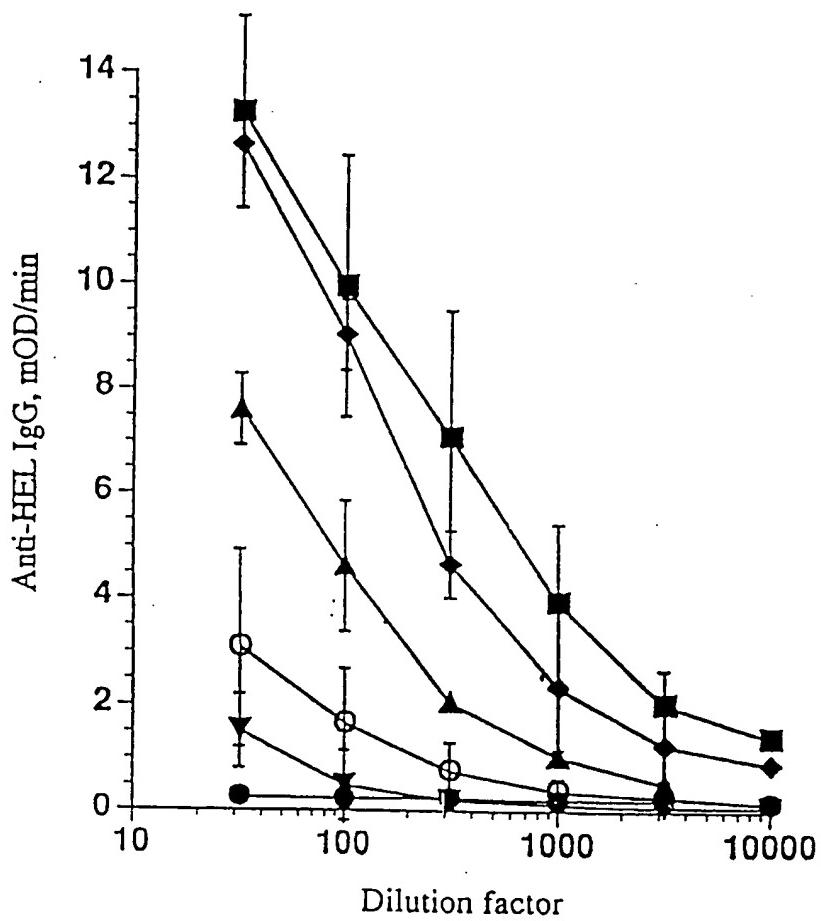
FIG. 7



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FIG. 8



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FIG. 9A

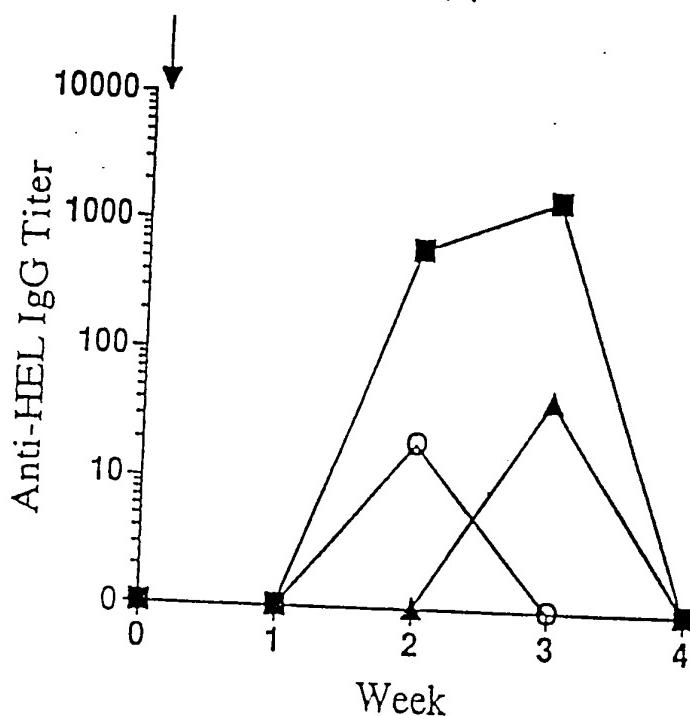
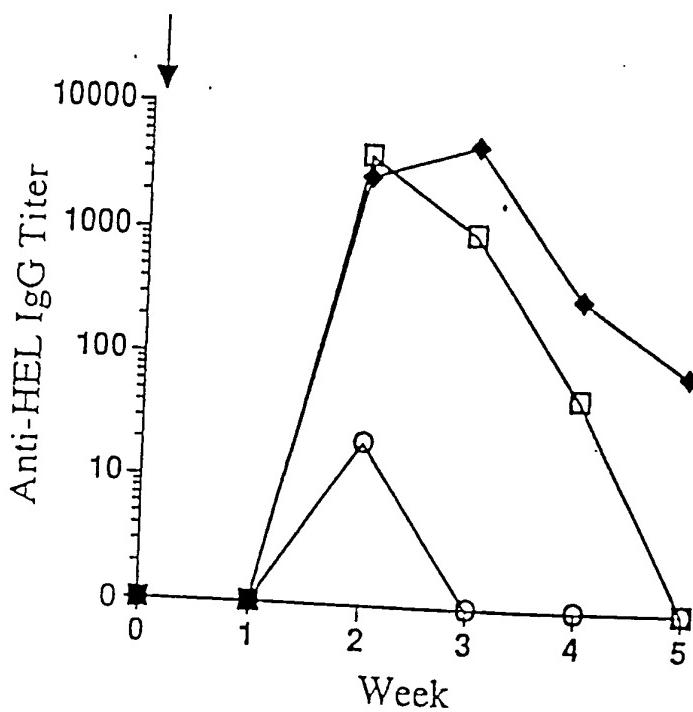


FIG. 9B

**SUBSTITUTE SHEET**

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FIG. 10

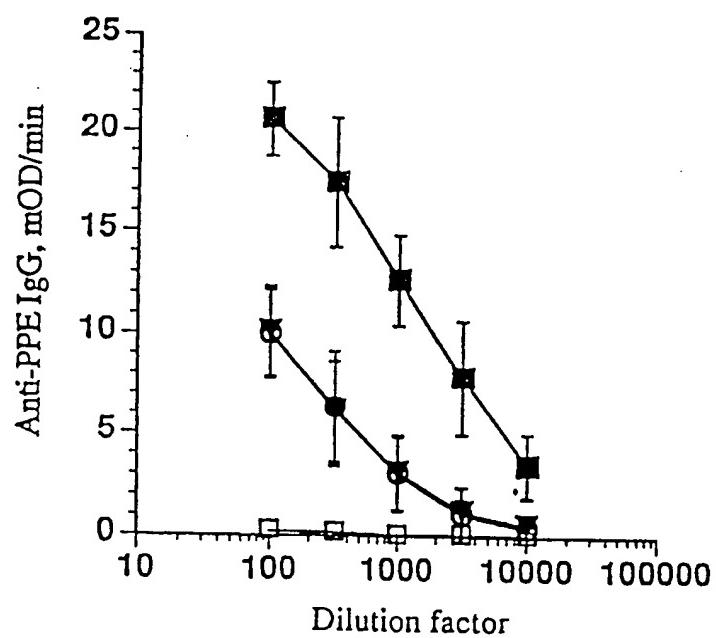
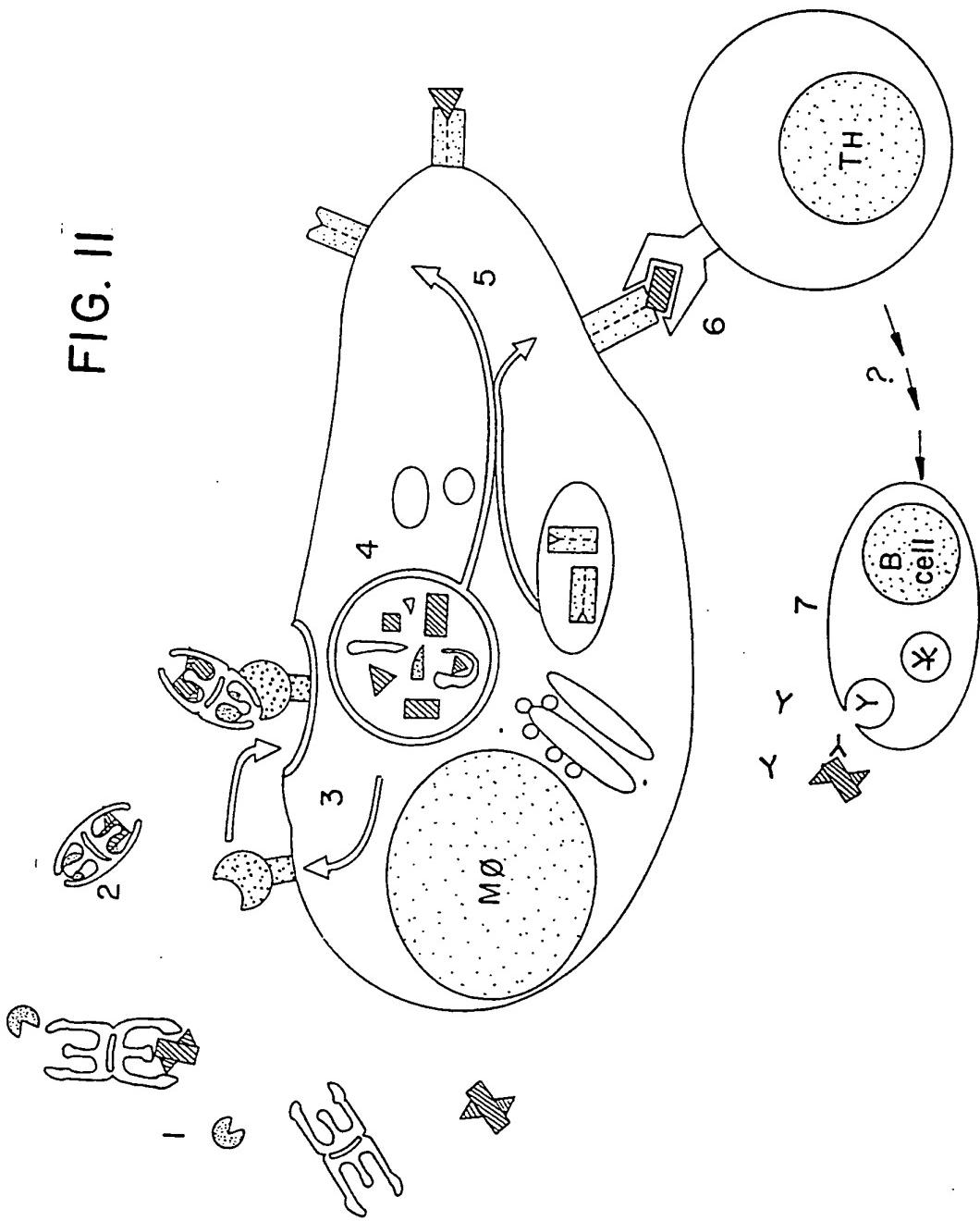


FIG. II



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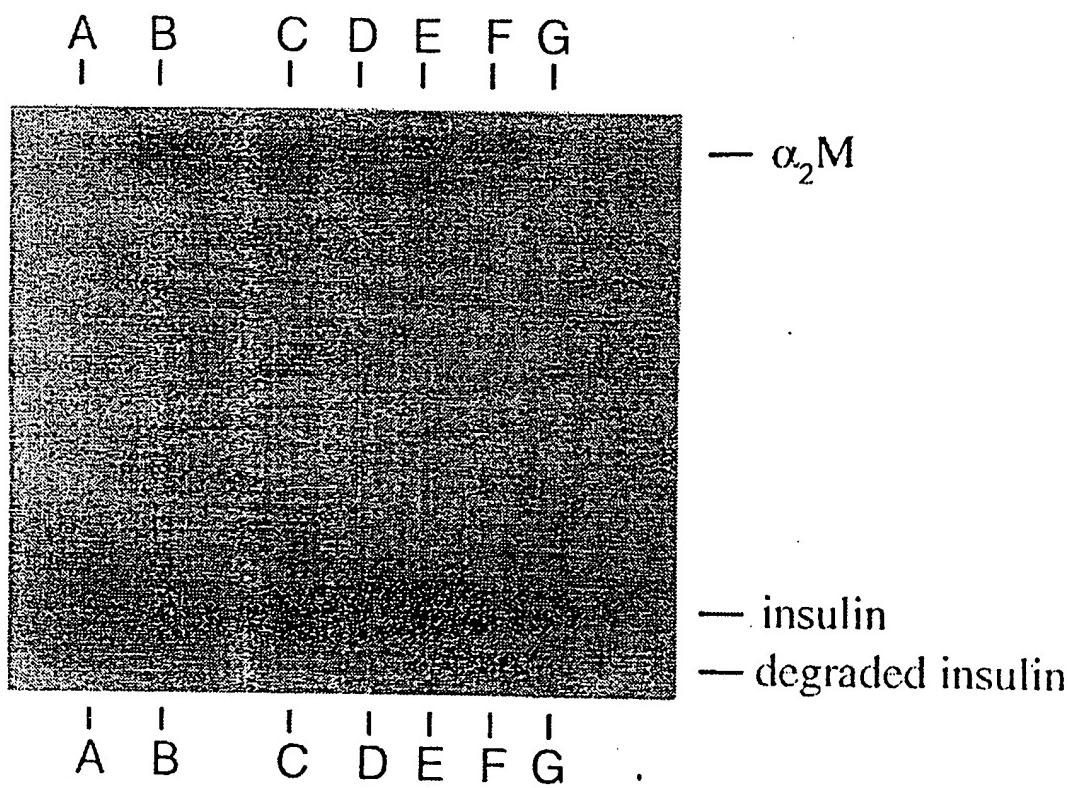


FIG. 12

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FIG. 13A

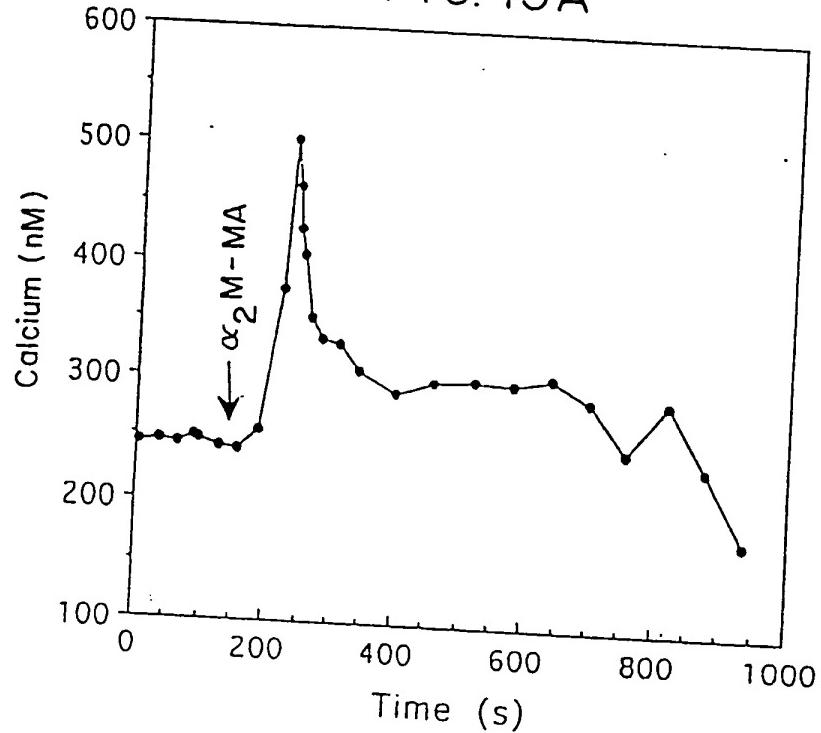
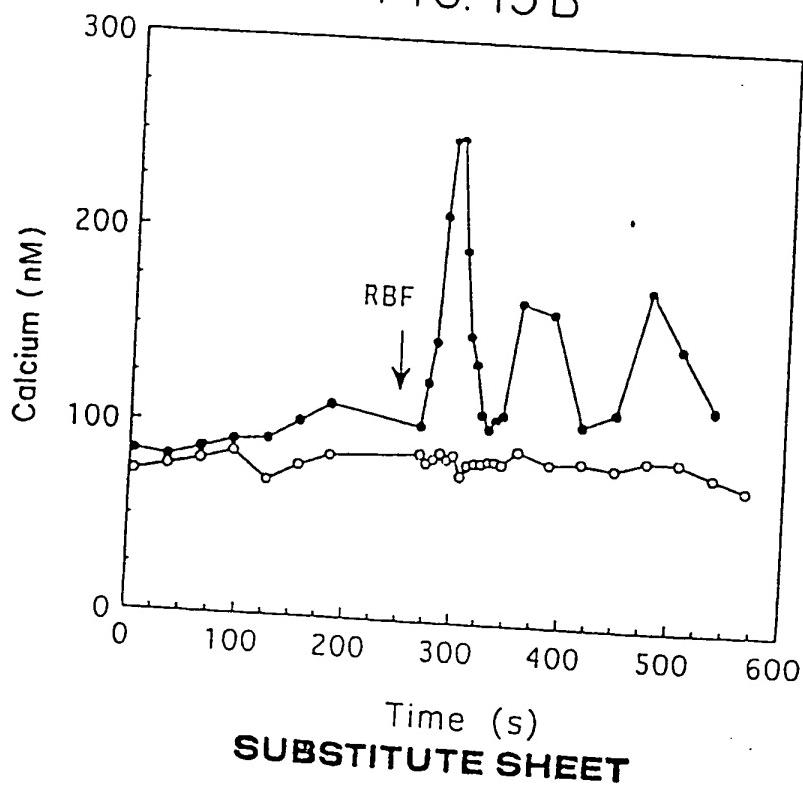


FIG. 13B

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FIG. 14

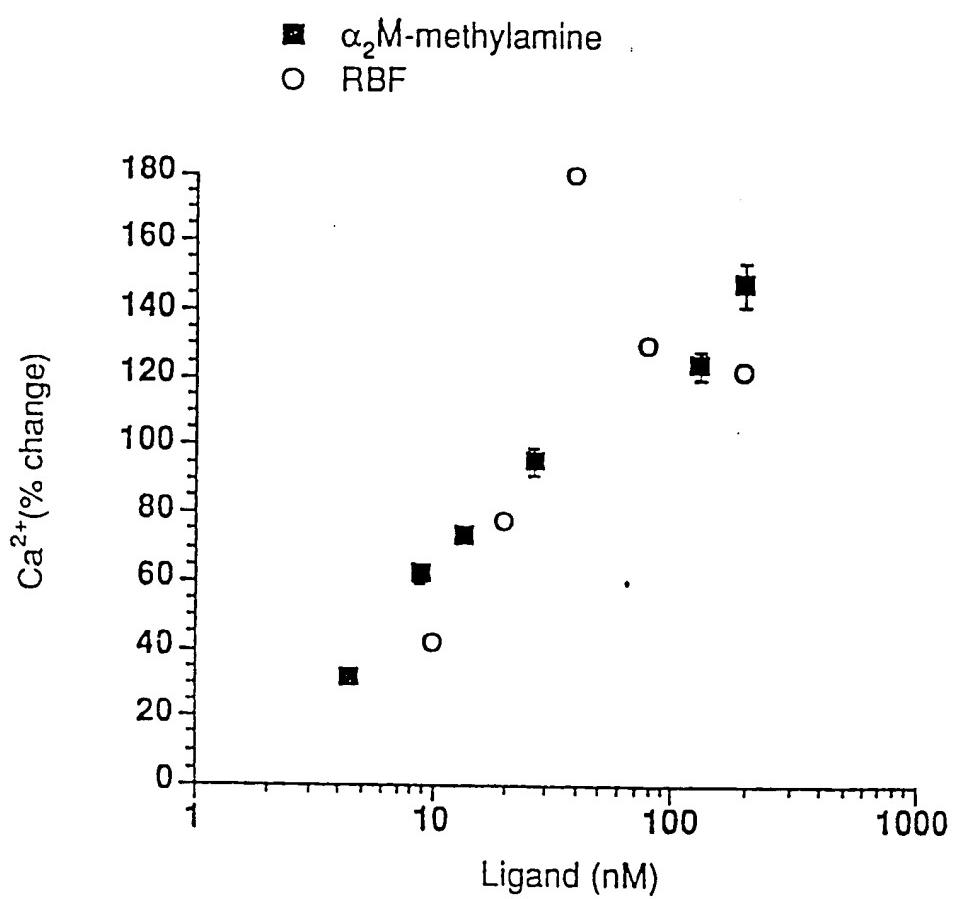


FIG. 15A

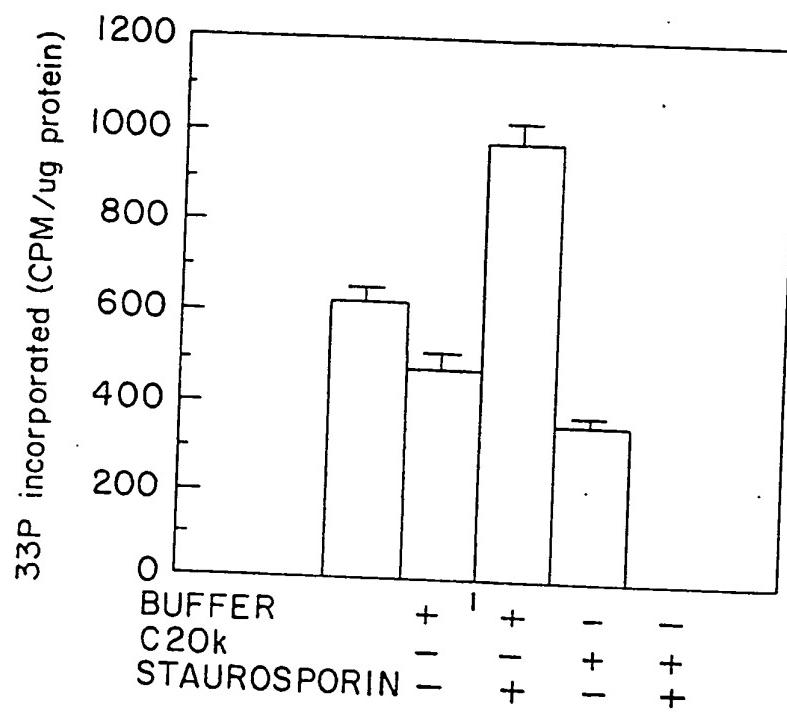
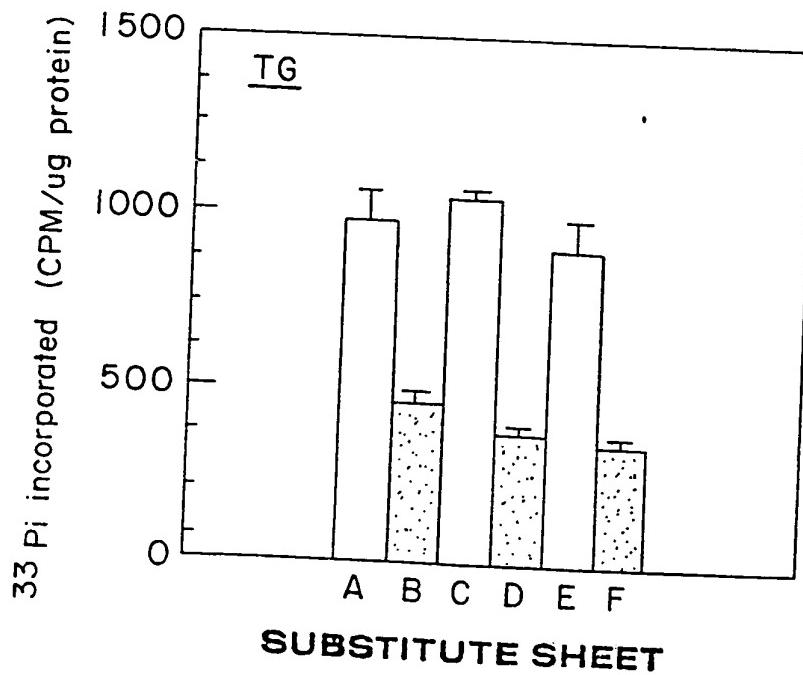


FIG. 15B



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FIG. 16A

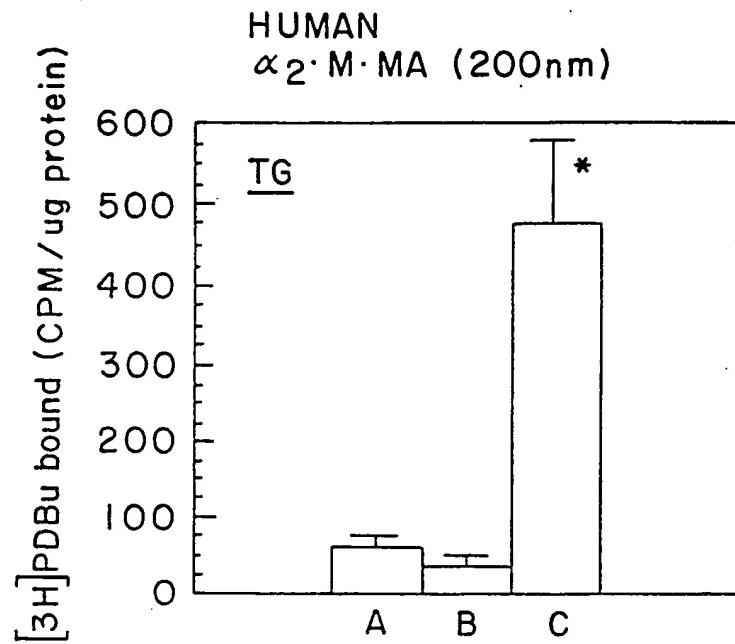
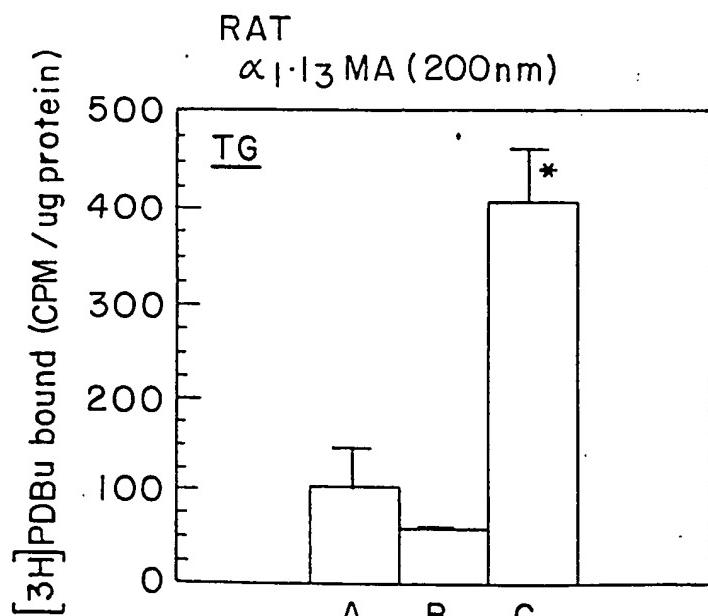
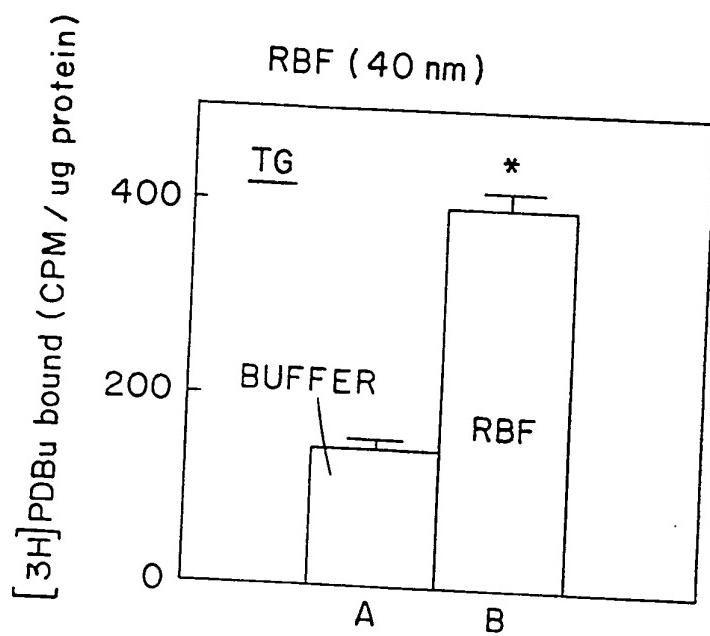


FIG. 16B



SUBSTITUTE SHEET

FIG. 16C



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 93/12479

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12P21/08 A61K47/48 G01N33/569 C12N5/00 A61K39/395
A61K35/14

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C12P G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO, A, 92 07003 (UNIVERSITY OF GUELPH &amp; UNIVERSITY OF VIRGINIA ALUMNI PATENT FOUNDATION) 30 April 1992 see claims</p> <p>---</p> <p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 150, no. 2, 29 January 1988, ORLANDO, FL, USA pages 883 - 889</p> <p>T. OSADA ET AL. 'Antibodies against viral proteins can be produced effectively in response to the increased uptake of alpha2-macroglobulin:viral protein conjugate by macrophages.' cited in the application see abstract</p> <p>---</p> <p>-/-</p>	1-3, 8-10, 18, 33, 37
X		1-4, 7, 8, 16-23, 31, 33, 34

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

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- \*'E' earlier document but published on or after the international filing date
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- \*'P' document published prior to the international filing date but later than the priority date claimed

- \*'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search  16 May 1994	Date of mailing of the international search report  01-06-1994
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer  Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 93/12479

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 146, no. 1 , 15 July 1987 , ORLANDO, FL, USA pages 26 - 31 T. OSADA ET AL. 'Murine T cell proliferation can be specifically augmented by macrophages fed with specific antigen.' cited in the application see abstract ---	1-4, 8, 18-23, 29, 33
A	THE JOURNAL OF IMMUNOLOGY vol. 142, no. 2 , 15 January 1989 , BALTIMORE, MD, USA pages 629 - 635 C. MUNCK PETERSEN ET AL. 'Immunosuppressive properties of electrophoretically "slow" and "fast" form alpha2-macroglobulin.' see abstract -----	1-3, 8, 18-20, 29

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/12479

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 1-15 (partially, as far as an *in vivo* method is concerned) and 33-35 (completely) are directed to a method of treatment of the Human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

## Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

**PCT/US 93/12479**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9207003	30-04-92	NONE	